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Avances en el estudio de cilindrospermopsina: detección de cianobacterias potencialmente productoras de la toxina y prospección de las etapas iniciales de su síntesis

Advances in the study of cylindrospermopsin: detection of potentially cylindrospermopsin-producing cyanobacteria, and prospect for the initial steps of the toxin synthesis

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A Djía y Hanwool

A mis padres y hermanos

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Acronyms and abbreviations

ABC	ATP binding cassette
Adda	(2*S*, 3S, 8S, 9S, 4E, 6E)-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyl-4, 6-decadienoic acid
AMDT	Amidinotransferase
amu	Atomic mass unit
ANOVA	Analysis of variance
Arg	Arginine
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BLAST	Basic local alignment search tool
BMAA	β-methylamino l-alanine
bp	Basepair
BSA	Bovine serum albumin
Chl_a	Chlorophyll a
Cm	Cloramphenicol
Ct	Cycle treshold
CyanoHAB	Cyanobacterial Harmful Algal Bloom
CYN	Cylindrospermopsin
CYN^-	CYN non-producing cyanobacteria
CYN^+	CYN producing cyanobacteria
Da	Dalton
D-Ala	D-alanine
DDCt	Delta delta cycle treshold
Deoxy-CYN	Deoxy-cylindrospermopsin
D-Glu	D-glutamate
D-MeAsp	D-methyl aspartate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked ImmunoSorbent Assay
ESI	Electrospray ionization
GAA	Guanidinoacetate
GABA	γ-aminobutyric acid
GF/F	Glass fiber filter
Gly	Glycine
GST	Glutathione S transferase
HAB	Harmful Algae Bloom
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High resolution liquid chromatography
Ip	Isoelectrical point
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobase
kDa	Kilodalton
Km	Kanamycine
K_m	Michaelis constant
kV	kilovolt
LB	Lysogeny Broth
Leu	Leucine

LPS	Lipopolysacharide
MALDI-TOF	Matrix laser desorption ionization time of flight
MAO	Madrid *Aphanizomenon ovalisporum*
MC^-	MC non-producing cyanobacteria
MC(s)	Microcystin(s)
MC^+	MC producing cyanobacteria
Mdha	Methyl dehydroalanine
mRNA	messenger RNA
MS	Mass spectrometry
N	Nitrogen
Nd	Not detected
NMR	Nuclear magnetic resonance
NRPS	Non ribosomal peptide synthetase
nt	Nucleotide
O.D.	Optical density
ORF	Open reading frame
P	Phosphorous
PAR	Photosynthetically active radiation
PBS	Phosphate buffer saline
PCC	Pasteur culture collection
PCR	Polymerase chain reaction
PDA	Photo diode array
P_i	Inorganic phosphorous
PKS	Polyketide synthase
PP	Protein phosphatase
PP1	Protein phosphatase type 1
PP2A	Protein phosphatase type 2A
PPI	Protein phosphatse inhibition
Psi	Pounds per square inch
PSII	Photosystem II
PSP	Paralytic shellfish poison
q PCR	quantitative PCR
Q-TOF	Quadrupole time of flight
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	ribosomic RNA
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TB	Tris buffer
U	Enzyme unit
UAM	Universidad Autónoma de Madrid
UTEX	University of Texas
V	Volt
V_{max}	Maximum velocity
WHO	World Health Organisation

SUMMARY/RESUMEN

SUMMARY

The occurrence of toxic cyanobacterial blooms is increasing world-wide in the last decades. A good number of cyanotoxins have been already described, the best known among them being microcystins (MC) and cylindrospermopsin (CYN), due to their high abundance and global distribution. It is frequent to find in blooms mixed populations of cyanobacteria producing MCs and CYN. In many cases, the known cyanotoxins contained cannot explain by themselves the toxicity of the blooms; therefore, other toxic compounds are to be present.

In this work, different approaches were used to get insight into cyanotoxicity: i) a new methodology was developed to detect simultaneously CYN and MC-producing strains; ii) a new cyanotoxic candidate has been found to accumulate in cyanobacteria, guanidinoacetate (GAA); iii) the enzyme responsible for the first reaction of CYN synthesis pathway has been characterized in a strain of *Aphanizomenon ovalisporum*; and iv) in this strain, it was analyzed the relationship between the production of CYN and the expression of some key genes considered to codify enzymes of CYN synthesis.

Several studies have revealed that the genes *mcy* and *aoa/cyr* are involved in MC and CYN synthesis, respectively. Based on the identification of those gene sequences, we developed a multiplex PCR assay that allows simultaneous detection of CYN^+ and MC^+ strains in mixed populations of cyanobacteria. For that, various primer sets were designed, by using *mcy* and *aoa* gene sequences. Purified DNA as well as cultured cell mixtures and field samples with MC and CYN-producing strains were suitable as DNA template. The method could be applied to environmental samples, implying a rapid and low-cost test to jointly detect the presence of CYN^+ and MC^+ cyanobacteria in sestonic fractions of water samples.

In the last years, several CYN^+ strains of *A.ovalisporum* have been isolated in water bodies from different geographical regions, leading to a rise in ecological and health risks. According to the models proposed, an amidinotransferase (AMDT) codified by the *aoaA* gene is the first enzyme involved in CYN synthesis. We have cloned and overexpressed the *aoaA* gene from the CYN^+ strain *A.ovalisporum* UAM-MAO, isolated in the laboratory. The recombinant purified AoaA was biochemically characterized, confirming that it is amidinotransferase. AoaA is similar in many aspects to the previously reported AMDT of *Cylindrospermopsis raciborskii*, CyrA: high substrate specificity for Arg and Gly, and a mixed sequential/ping-pong kinetic

mechanism in its activity. We have further observed that AoaA is inhibited by GAA in a non-competitive manner.

GAA is a precursor of CYN, being formed in the first step of the toxin synthesis pathway. On the other hand, it is one of the most extensively studied toxic guanidine compounds. GAA changes can affect the nervous system and induce hyperhomocysteinemia, representing a risk factor for cardiovascular diseases. In spite of the evidence supporting GAA toxicity and its role in CYN synthesis, no data have been reported on the accumulation of GAA in any cyanobacterium. We have analyzed and compared the content of GAA in cultures of diverse cyanobacteria types, both CYN-producing (CYN^+) and non-producing (CYN^-). GAA was present in the majority of the strains tested, although the highest content was found in the CYN^+ strain, *A. ovalisporum* UAM-MAO. Therefore, GAA appears to be a general cyanobacterial metabolite that due to its proven toxic should be considered when studying cyanobacterial toxicity, and in cyanotoxicity management.

In the last years, several highly homologous gene clusters related to CYN synthesis, *aoa* and *cyr*, have been described in different cyanobacteria genera. We have studied *aoaA-C* and *cyrJ* gene expression by real time qPCR in *A. ovalisporum* UAM-MAO grown under optimal conditions. A good correlation between the expression of those genes and CYN production was found. Furthermore, taking into account the CYN high nitrogen content, a possible relation of the nitrogen master controller NtcA with CYN synthesis was explored, by following the expression kinetics of the codifying gene *ntcA*. The tendency of *ntcA* relative expression looks similar to that of *aoaA-C* and *cyrJ*. Besides, three putative NtcA binding sites have been localized within the *aoaA-C* sequence. These findings support the idea of CYN production be regulated by NtcA.

Since arginine and glycine seem to be the only substrates accepted by the AMDT related to CYN synthesis, we studied the effect of these amino acids in cultures of *A. ovalisporum* UAM-MAO. Arg clearly caused an increase in CYN, but Gly a decrease. The decrease appears not to result from inhibiting the activity of the genes considered to be involved in CYN synthesis, since Gly, as Arg, enhanced the transcription of *aoaA-C* and *cyrJ* genes. On the other hand, culture growth was affected by Arg and Gly in a similar way to CYN production, Arg stimulating and Gly impairing it. Therefore, the influences of both Arg and Gly on CYN oscillations during growth seem not to be due

to a specific effect on the first step of CYN synthesis, but to changes in the physiological cell status.

RESUMEN

En las últimas décadas está incrementando mundialmente la aparición de afloramientos masivos (*blooms*) de cianobacterias tóxicas. Ya se ha descrito un gran número de cianotoxinas. Entre ellas, las microcistinas (MCs) y la cilindrospermopsina (CLP) son las mejor conocidas, debido a su elevada abundancia y a su distribución global. Es frecuente la aparición de *blooms* formados por poblaciones heterogéneas de cianobacterias productoras de MC (MC^+) y CLP (CLP^+). En muchos casos, las cianotoxinas conocidas presentes en los *blooms* no pueden explicar por si solas los episodios de cianotoxicidad y, por tanto, otros compuestos tóxicos deben contribuir a la cianotoxicidad.

En este trabajo se ha tratado varios aspectos de cianotoxicidad mediante diferentes abordajes experimentales: i) se ha desarrollado una nueva metodología para detectar simultáneamente cepas potencialmente productoras de MCs y CLP; ii) se ha registrado la presencia en cianobacterias de guanidinoacetato (GA), que podría ser una nueva cianotoxina; iii) se ha caracterizado la enzima responsable de la primera reacción de la vía de síntesis de la CLP en una cepa de *Aphanizomenon ovalisporum*; y iv) en esta cepa se ha estudiado la relación entre la producción de toxina y los niveles expresión de algunos genes implicados en su síntesis.

Diversos estudios genéticos han mostrado que los genes *aoa/cyr* y *mcy* están implicados en la síntesis de CLP y MCs, respectivamente. Tomando como base este hecho, se ha desarrollado un método de PCR múltiple para poder detectar simultáneamente cepas potencialmente CLP^+ y MC^+ en poblaciones heterogéneas de cianobacterias mediante la identificación de varias secuencias *aoa/cyr* y *mcy*. Para ello, se diseñó un conjunto de cebadores específicos. Como molde de ADN sirvieron tanto ADN purificado como células de cultivo y muestras de campo con cepas MC^+ y CLP^+. La aplicación a muestras naturales, posibilitaría un diagnóstico rápido y económico para detectar simultáneamente la presencia de cianobacterias potencialmente MC^+ y CLP^+ en la fracción sestónica de muestras de agua.

En los últimos años, el aislamiento de varias cepas de *Aphanizomenon ovalisporum* CLP^+ en diferentes zonas del mundo ha motivado que esta especie sea considerada como un serio peligro ecológico y sanitario. De acuerdo con los modelos propuestos, la primera enzima de la vía de síntesis de la CLP es una amidinotransferasa (AMDT), codificada por el gen *aoaA*. Se ha clonado y sobreexpresado este gen de una cepa de *A. ovalisporum* CLP^+ aislada en el laboratorio. Posteriormente se ha caracterizado bioquímicamente la proteína AoaA, habiéndose confirmado su actividad de L-arginina: glicina amidinotransferasa. La actividad de AoaA muestra un mecanismo de tipo mixto (ping-pong/secuencial) y se inhibe no-competitivamente por el producto de la reacción, guanidinoacetato. Además, AoaA conserva los residuos del centro catalítico y la especificidad de sustrato descritos anteriormente en la AMDT *CyrA* de *Cylindrospermopsis raciborskii*.

Como se ha mencionado, el GA es un precursor de la CLP, producto de la primera reacción de la síntesis de la toxina. Por otra parte, es uno de los compuestos guanidínicos tóxicos más estudiados. Cambios en su concentración pueden afectar al sistema nervioso, inducir hipercisteinemia, representando un riesgo cardiovascular. A pesar de esas pruebas experimentales, no se ha descrito la acumulación de este compuesto en cianobacterias. Hemos analizado y comparado el contenido de GA en varias cepas de cianobacterias productoras (CLP^+) y no productoras de CLP (CLP^-). El GA se acumulaba en la mayoría de cianobacterias analizadas, si bien la concentración más alta fue la de la estirpe CLP^+ *A. ovalisporum* UAM-MAO. Por tanto, el GA parece ser un metabolito común de cianobacterias y que debido a su carácter tóxico debería ser tenido en cuenta en los estudios y gestión de la cianotoxicidad.

En los últimos años, se ha descrito varios agrupamientos génicos de distintas cianobacterias relacionados con la síntesis de CLP, todos ellos con un alto grado de homología en sus secuencias génicas. Hemos estudiado la expresión de los genes *aoaA-C* y *cyrJ* mediante PCR cuantitativa en la estirpe *A. ovalisporum* UAM-MAO, cultivada en condiciones óptimas. Se ha encontrado una buena correlación entre los niveles de expresión génica y la producción de CLP. Además, teniendo en cuenta el alto contenido en nitrógeno de la molécula de CLP, se ha explorado la posible relación del controlador global del metabolismo del nitrógeno (NtcA) con la producción de toxina, analizando la cinética del nivel de expresión de *ntcA*.

La tendencia de la cinética de expresión de los genes *aoa/cyr* y de *ntcA* coinciden con la de producción de la toxina. Además, se ha localizado tres posibles lugares de unión de NtcA en el agrupamiento génico *aoa* de *Aphanizomenon ovalisporum.* Estos resultados sugieren una posible participación de NtcA en el control de la producción de CLP.

Puesto que arginina (Arg) y glicina (Gli) parecen ser los dos únicos sustratos aceptados por la AMDT implicada en la síntesis de CLP, estudiamos el efecto de estos dos aminoácidos en cultivos de *A. ovalisporum* UAM-MAO. En líneas generales, la Arg produjo un aumento del contenido de CLP, mientras que la Gli causó el efecto contrario. Este efecto de la Gli no parece estar asociado a una inhibición de la expresión de los genes relacionados con la síntesis de CLP ya que, al igual que la Arg, todos los genes analizados aumentaron su expresión en presencia de los aminoácidos. Por otro lado, el crecimiento se vio afectado de igual modo que la producción de tóxina. Así, mientras la Arg estimulaba el crecimiento la Gli lo inhibía. Por lo tanto, las fluctuaciones de CLP causadas por la presencia de Arg y Gli no parecen estar relacionados con el primer paso de la síntesis de la toxina sino con variaciones en el estado fisiológico de la cianobacteria.

1. INTRODUCTION

INTRODUCTION

1.1. General aspects of cyanobacteria

Cyanobacteria are an ancient group (3.5 billion years) of gram-negative bacteria that have played a key role in changing the composition of the terrestrial atmosphere. They perform oxygenic photosynthesis, being important contributors to global oxygen production. Besides, they are among the most significant groups in earth biogeochemical cycles, especially carbon and nitrogen.

Cyanobacteria are crucial primary producers, incorporating organic matter to the ecosystems. Even though all cyanobacteria are photoautotrophic, some are able to live photoheterotrophically or even heterotrophically in the dark. Due to their high metabolic versatility and the ability to form specialized cells, they can be found in a wide range of aquatic and terrestrial habitats (Whitton and Potts, 2000), even in extreme environments as, volcanic (Ward and Castenholz, 2000) or desert areas (Wynn-Williams, 2000), polar latitudes (Vincent, 2000) and highly polluted systems (Radwan and Al-Hasan, 2000). Among specialized cells, heterocysts are formed by some filamentous cyanobacteria for atmospheric nitrogen fixation in the absence of combined nitrogen. Heterocysts provide an anoxic environment for the enzyme nitrogenase that catalyzes N_2 fixation and is irreversibly inhibited by oxygen. Moreover, under stress conditions, vegetative cells of some filamentous cyanobacteria can differentiate into resistant spores called akinetes, which can germinate under favorable environmental conditions. Other specialized cells are motile filaments called hormogonia that are involved in asexual reproduction and colonization.

Among the special features for cyanobacteria success in aquatic ecosystems are the ability of many species to form gas vacuoles, and their capability to utilize different kinds of light, thanks to the various pigment-protein complexes that form part of the photosynthetic antenna (Fig.1.1). The major antenna complex in cyanobacterial antenna is organized in the structures called phycobillisomes which are formed by different association of proteins and pigments (phycobilins) anchored to the thylakoid membranes. Each pigmented protein (phycobilliprotein) shows a specific absorption maximum in the visible light spectrum (450-660 nm) covering parts of the light not absorbed by the chlorophyll a, from the antenna or mainly from the reaction center. Both the existence of gas vacuoles and the characteristic antenna give to cyanobacteria a

considerable advantage against their competitors in aquatic systems, allowing them to settle in the optimum area along the water column. Those advantages are part of the basis for bloom formation (Oliver and Ganf, 2000). On the other hand, a good number of cyanobacteria can grow in symbiosis with other prokaryotic and eukaryotic organisms, such as fungi, animals or plants (Whitton and Potts, 2000), providing energy or inorganic compounds to the host.

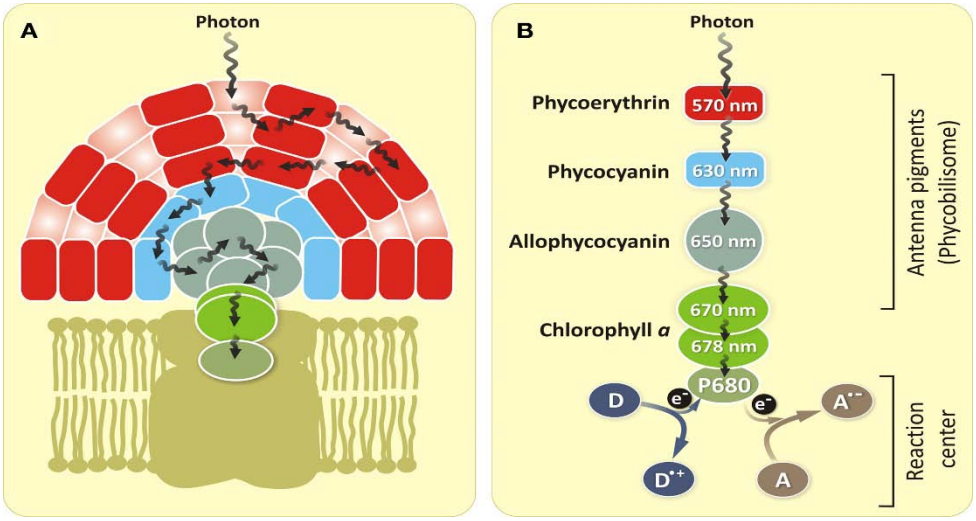


Fig.1.1. Structural organization and protein-pigment composition of the cyanobacterial PSII antenna (A); and energy transfer steps including charge separation (photochemical reaction) at the PSII photoreaction center (B). (Source: www.frontiersin.org).

1.1.1. Taxonomy of cyanobacteria

Cyanobacteria constitute one of the largest groups of prokaryotes, and its classification is not easy. For this reason, it is permanently under revision, and constantly updated. Some features, such cell shape, presence and form of specialized cells and type of reproduction, among others, have been considered for taxonomy. The bacteriological classification of Rippka et al. (1979), and the botanical approximation proposed by Komarek and Anagnostidis (1989), as well as other subsequent cyanobacteria sorting attempts agree in establishing two main groups, unicellular (non-filamentous) and filamentous, divided in different sections Table 1.1.

Table 1.1. Classification of cyanobacteria (adapted from Rippka, 1979)

<table>
<tr><td rowspan="2">Unicellular; single cells or forming colonial aggregates</td><td colspan="3">Reproduction by binary fission or budding
Section I
Chroococcales</td></tr>
<tr><td colspan="3">Reproduction by multiple fission; baeocytes forming
Section II
Pleurocapsales</td></tr>
<tr><td rowspan="3">Filamentous; chain of cells with intercalary cell division</td><td rowspan="3">Reproduction by random trichome breakage, hormogonia and/or akinete germination</td><td>Trichomes composed only of vegetative cells</td><td>One plane division
Section III
Oscillatorialles</td></tr>
<tr><td rowspan="2">Potentially heterocysts and akinetes formers</td><td>One plane division
Section IV
Nostocales</td></tr>
<tr><td>More than one plane division; truly branched trichomes
Section V
Stigonematales</td></tr>
</table>

1.1.2. Cyanobacterial blooms

Favorable environmental conditions can promote a vast growth of cyanobacteria in both freshwater and marine habitats (Fig.1.2), forming blooms. These blooms cause an important impact in water quality, such as increase of turbidity and oxygen depletion, causing serious hazard for other aquatic organisms.

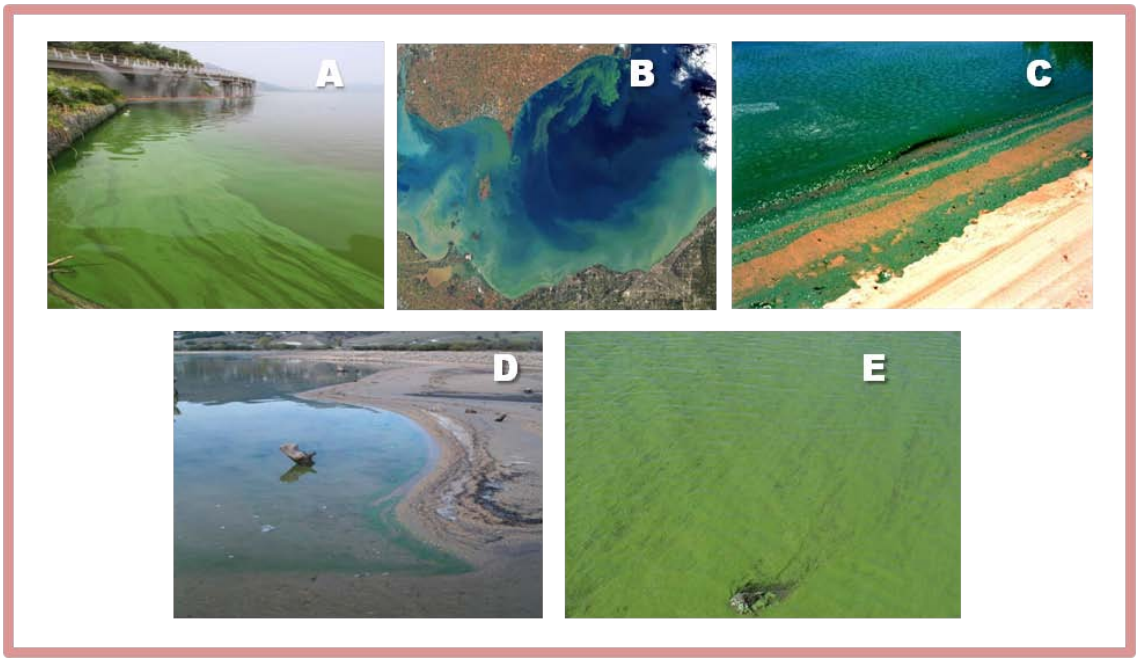


Fig.1.2. Blooms from different geographical regions. A) Nakdonggang River (South Korea); source: Yonhap news. B) Lake Erie (Canada and USA); source: NASA; C) Matilda Bay (Western Australia); photo by Tom Rose (WA, Waters and Rivers Commission). D and E) Pinilla reservoir (Madrid, Spain); photo by FF del Campo (UAM).

In the last decades the occurrence of cyanobacterial blooms have increased, partly as a consequence of water eutrophication (O'Neil et al., 2012) and probably climate change (Paerl and Huisman, 2008). Over-enrichment of waters (principally by nitrogen and phosphorous rich compounds), mainly by human activities (agriculture, industrial and urban waste), has caused a global spreading out of cyanobacterial blooms (Fig.1.3). Especially important are the blooms formed by cyanobacteria that produce toxic compounds, known as “cyanoHABs” (Carmichael, 2001; Paerl and Huisman, 2008,). Frequently, in those toxic blooms appeared mixed populations of cyanobacteria species, including toxic and non-toxic strains (Fastner et al., 2007; Al-Tebrineh et al., 2012).

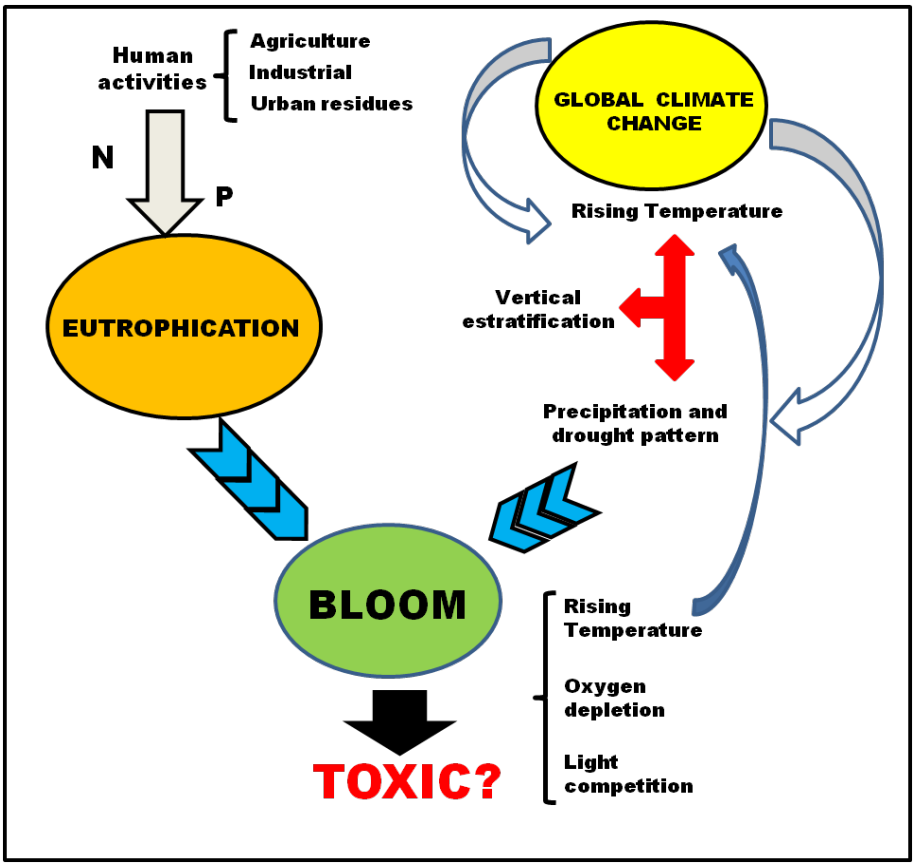


Fig.1.3. Some factors affecting bloom formation.

1.2. Cyanobacterial toxins

One of the most interesting qualities of cyanobacteria is the wide range of bioactive compounds that are able to synthesize, such as anticancer agents, antiviral or ultraviolet protectors (Sivonen and Börner, 2008). More than 40 cyanobacteria genera belonging to all taxonomic sections are represented as potential producers of bioactive compounds (Carmichael, 2001). Such features make cyanobacteria be potentially useful in many socio-economic sectors, as agriculture (Prasanna et al., 2010) and pharmaceutical industry (Chlipala et al., 2011). But, some of those bioactive products also represent a health hazard for many organisms, including human. They constitute a large group of noxious substances called cyanotoxins. Nowadays, cyanobacteria overgrowth linked to toxin production are important issues in water management.

There are different cyanotoxin classifications, depending on the chemical structure or biological properties. A traditional classification establishes a relation between the compound and its toxic action (Table 1.2). It is important to remark the incessant

appearance of new cyanobacteria toxic compounds, which obliges to a continuous update of cyanotoxin lists.

Table 1.2. Cyanobacterial toxins

TOXIN	COMPOUND	TOXIN- PRODUCING GENERA
Hepatotoxins	Microcystins (>90 variants)	*Microcystis, Anabaena, Nostoc, Planktothrix, Nodularia,Woronichinia*
	Nodularins	*Nodularia*
Neurotoxins	Anatoxin-a	*Anabaena, Oscillatoria, Aphanizomenon, Cylindrospermum, Planktothrix*
	Saxitoxins	*Cylindrospermopsis, Anabaena, Aphanizomenon, Lyngbya*
	Homoanatoxin-a	*Anabaena*
	Anatoxin-a(s)	*Anabaena*
	β-methylamino L-alanine (BMAA)	*Nostoc*
Cytotoxins	Cylindrospermopsin	*Aphanizomenon, Cylindrospermopsis, Anabaena,Umezakia*
Irritants and gastrointestinal toxins	Aplysiatoxin Lyngbyatoxin LPS (lipopolysacharide)	*Anabaena, Planktothrix, Lyngbya, Microcystis*

Frequent toxicity episodes associated to harmful cyanobacterial blooms have been reported; being the first back in 1878 (Francis) and some of them are included in Table 1.3. The majority refer to wild and domestic animals events, but outbreaks of human poisonings have also been confirmed, as those in Palm Island disease (Australia), linked to cylindrospermopsin (CYN) (Griffiths and Saker, 2003), and even human deaths in Caruaru (Brazil), after exposure to microcystins (MCs) during a hemodialysis treatment (Azevedo et al., 2002).

Table 1.3. Examples of animal poisoning associated with cyanobacteria (adapted from Chorus and Bartram, 1999; WHO)

Country	Animals affected	Pathology	Organism associated	Reference
Argentina	Cattle	Hepatotoxicity (Microcystin)	*Microcystis aeruginosa*	Odriozola et al., 1984
Australia	Sheep	Hepatotoxicity (Microcystin)	*Microcystis aeruginosa*	Jackson et al., 1986
Australia	Sheep	Neurotoxicity (PSPs)	*Anabaena circinalis*	Negri et al., 1995
Australia	Cattle	Hepatoxicity (Cylindrospermopsin)	*Cylindrospermopsis raciborskii*	Thomas et al., 1998
Canada	Cattle	Neurotoxicity (anatoxin-a)	*Anabaena flos-aquae*	Carmichael and Biggs, 1978
Finland	Waterfowl, fish, Muskrats	Hepatoxicity and gill damage (Microcystin)	*Plankthotrix agardhii*	Eriksson et al., 1986
Scotland	Fish	Gill injury (Microcystin)	*Microcystis aeruginosa*	Bury et al., 1995
USA	Dogs	Neurotoxicity (anatoxin-a(S))	*Anabaena flos-aquae*	Mahmood et al., 1988
Netherlands	Dogs	Neurotoxicity ((homo)-anatoxin-a)	*Phormidium sp.*	Faassen et al., 2012
USA	Dogs	Hepatotoxicity (Microcystin)	*Microcystis aeruginosa*	Van der Merwe et al., 2012
Switzerland	Cattle	Hepatoxicity and Neurotoxicity (Microcystin and Anatoxin-a)	*Oscillatoria limosa* *Oscillatoria tenuis*	Mez et al., 1997
South Africa	Livestock	Hepatotoxicity (Nodularin and Microcystin)	*Nodularia spumigena* *Microcystis aeruginosa*	Van Halderen et al., 1995

MCs and CYN are the most representative cyanotoxins (Falconer and Humpage, 2005 and 2006), due to their implications in the majority of cyanotoxin occurrences reported so far, and to their wide distribution.

Cyanotoxins are strain specific and not essential compounds for cyanobacterial life hence, they are considered as products of secondary metabolism (Sivonen and Jones, 1999). To date, no physiological role of cyanotoxins has been demonstrated, but many hypotheses have been proposed trying to explain their presence in aquatic ecosystems.

Thus, it has been claim a role for MCs as, predator defense (Jang et al., 2003, Jang et al., 2008), allelopathic effectors (Leflaive and Ten-Hage, 2007) and iron chelator (Utkilen and Gjolme, 1995; Sevilla et al., 2008; Alexova et al., 2011, Fujii et al., 2011). In the case of CYN, biological role as phosphate scavenger (Bar-Yosef et al., 2010; Kaplan et al., 2012) has been suggested.

1.2.1. Microcystins

1.2.1.1. Chemical structure

Microcystins (MCs) are the most studied group of cyanotoxins, due to its wide and frequent presence in diverse habitats. Their chemical structure is a cyclic heptapeptide with a common moiety formed by D-Alanine/ X/ D-methylaspartic/ Z/ (2*S*, 3*S*, 8*S*, 9*S*, 4*E*, 6*E*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (Adda)/ D-glutamate and N-methylhydroalanine (Fig.1.4). The unusual amino acid Adda, closely related to MC toxicity, has been also found in other cyanobacterial peptides including nodularin and motuporin. More than 90 MCs variants have been described (del Campo and Ouahid, 2010), with a molecular mass ranging between 900 and 1117. The number of MCs is permanently updated. Different chemical modifications could appear in every single residue, but L-amino acids, X and Y, in the positions 2 and 4 of the heptapeptide, are the main responsible for the great MC variety.

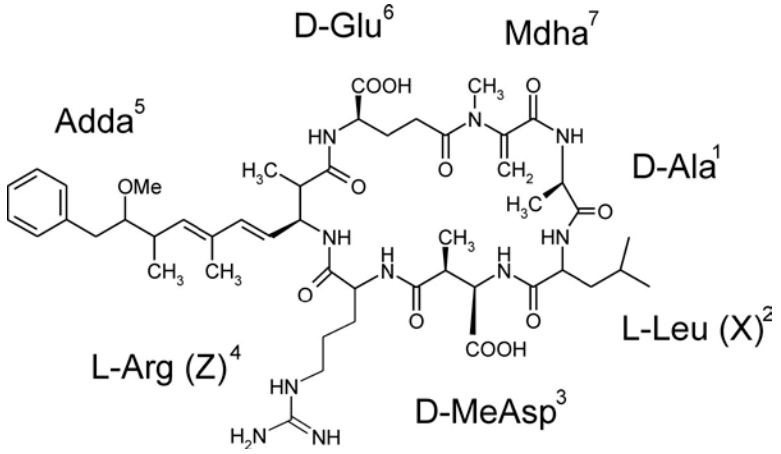


Fig.1.4. Chemical structure of microcystin-LR. Positions 2(X) and 4(Z) are variable L-amino acids.

The methodology to analyze MCs is diverse. For a rapid analysis the most usually method is immunochemical, ELISA. Its value is limited, because some MCs react poorly against the antibodies of MC-LR that the method utilizes. Also, a rapid methodology is that based on the ability of MCs to inhibit protein phosphatases (PP). The advantage of this colorimetric method is that it allows quantifying the overall MC activity in the samples, rather than the MC amount. It is suitable mainly for aqueous samples. Other methodology requires more robust and higher-price equipments, often not available in ordinary water management laboratories: HPLC, HPLC-MS, MALDI-TOF MS or NMR.

1.2.1.2. Microcystin toxicity

Most of the experiments on MCs toxicity have been mainly carried out using mice or hepatocyte cultures. The most common acute effects occur in liver, with manifest significant histological changes (Jochimsen et al., 1998). Besides, long-term MCs exposure has been associated to hepatocarcinogenesis (Yu et al., 2001; Svircev et al., 2009). MCs can be incorporated into hepatocytes by the bile acid organic anion transport system (Eriksson et al., 1990). Other tissues can also be affected by MCs, including kidney or timus (Suput, 2011). Moreover, MCs have been associated to apoptosis induction (Zegura et al., 2011) and neurotoxicity, and even have been related to Alzheimer disease (Li et al., 2012).

The level of toxicity is highly variable among different MCs, depending both on its chemical composition and route of exposure; but all of the variants evaluated seem to act as inhibitors of serine threonine protein phosphatases, of type 1 (PP1) and 2A (PP2A) (Mackintosh et al., 1990). MCs bind to the catalytic site of PPs, blocking the entrance of the substrate. By so doing, they increase phosphorylation in hepatocytes, and as a consequence, cytoskeleton disorganization occurs. The unusual amino acid Adda needs to be present for protein phosphatase inhibition (PPI), but it is not sufficient for toxicity (Gulledge et al., 2002).

MCs are also considered as health hazard compounds due to their accumulation in animals (Jiang et al., 2012a) and plants (Mohamed and Al Shehri, 2009; Peuthert and Pflugmacher, 2010; Pichardo and Pflugmacher, 2011). Therefore, MCs accumulation within the food web may constitute an important human health risk (Papadimitriou et al.,

2012) . Due to their worldwide distribution and their high toxicity, the World Health Organization (WHO) guidelines established a limit value of 1µg/L equivalent MC-LR for drinking water. Several countries have adopted this value in their legislation (Burch, 2008), including Spain (RD140/2003).

1.2.1.3. Genetics and synthesis

MCs are synthesized by microcystin synthetase, a large multimodular enzymatic complex similar to those involved in other cyanobacterial peptides and different products of other microorganisms, including antibiotics and immunosuppressants. Such enzymatic complexes contain non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) activities. NRPSs are able to work with proteinogenic and non-proteinogenic amino acids. This feature would allow synthesizing a huge variety of compounds, providing a great metabolic versatility in cyanobacteria. The minimal module of a NRPS complex bears three catalytic domains, putatively responsible for adenylation, thiolation and condensation activities. In addition, other tailoring activities such as O-methyl transferase, epimerization, N-methylation or oxidation domains can be present for further chemical modifications (Fig.1.5).

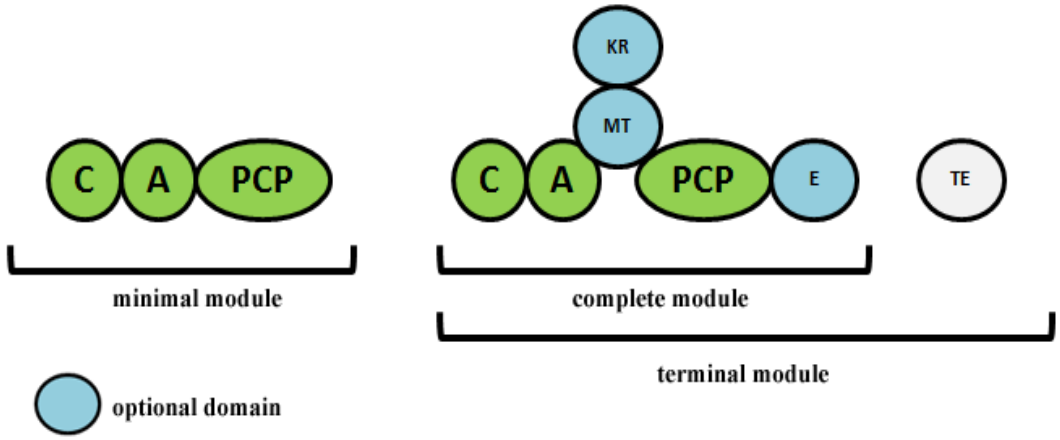


Fig.1.5. Non ribosomal peptide synthetase (NRPS) catalytic domains (modified from Kehr et al., 2011). Abbreviations: C, Condensation; A, Adenylation; PCP, Peptidyl carrier protein; KR, Ketoreductase; MT, Methyltransferase; E, Epimerase; TE, Thioesterase.

PKSs are also modules containing multi-domain enzymes, and consist of ketosynthase, acyltransferase, ketoreductase, dehydratase, enoyl reductase and acyl carrier protein (Fig.1.6).

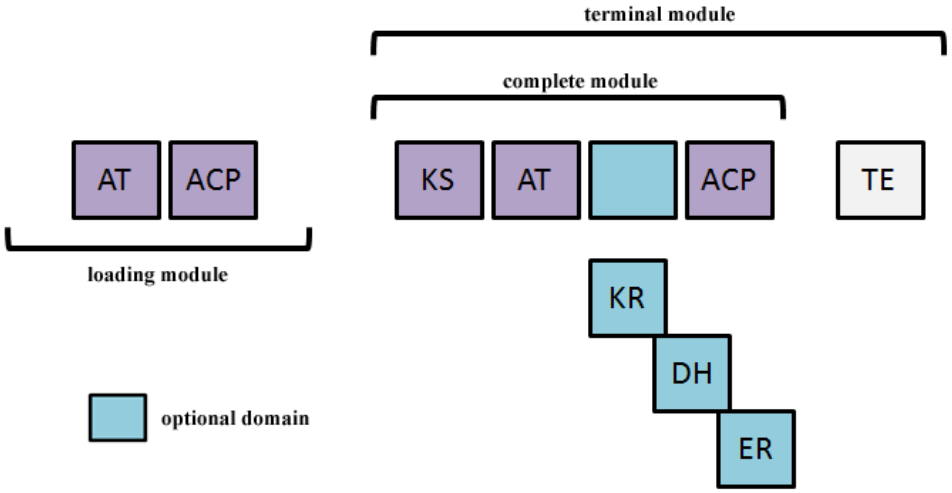


Fig.1.5. Polyketide synthase (PKS) catalytic domains (modified from Kehr et al., 2011). Abbreviations: AT: Acyltransferase; ACP: Acyl carrier protein; KS: Ketosynthase; KR: Methyltransferase; DH: Dehydratase; ER: Enoyl reductase; TE: Thioesterase.

Generally, NRPSs and PKSs are codified by genes responsible for multiple activities, which are clustered and organized in a characteristic manner to drive each step of the synthesis in a fixed order (Fig.1.7). Thus, the specific combination of modules establishes the structure and, hence, the activity of the final molecule. In cyanobacteria many genomes contain NRPS/PKS gene sequences, the most frequent being in large genomes, as those of *Anabaena*, *Microcystis* or *Planktothrix*.

MC synthetase genes were firstly described in *Microcystis aeruginosa* K-139 (Nishizawa et al., 1999), the corresponding genes were named *mcy*. The *Microcystis* genus is the most common MC producer, and it often forms blooms in freshwater water bodies. Moreover, the corresponding *mcy* genes of *Microcystis aeruginosa* PCC 7806 (Tillett et al., 2000), *Planktothrix agardhii* CYA 126 (Christiansen et al., 2003) and *Anabaena* sp. strain 90 (Rouhiainen et al., 2004) have also been completely sequenced.

MC biosynthesis clusters contain approximately 55 kb bearing 9-10 open reading frames (ORFs). NRPS and PKS activities are codified by *mcy*A to *mcy*E and *mcy*G; and further tailoring activities are encoded in genes *mcy*F, *mcy*H, *mcy*I, *mcy*J and *mcy*T. A high degree of sequence conservation in the *mcy* clusters characterized till moment has been reported (Fig.1.7). Other outstanding cyanotoxin, deeply related to MC, is the pentapeptide Nodularin. Its chemical structure is very similar to that of MC. Nodularin it is also synthesized via NRPS/PKS, and the codyfing gene regions (*nda* genes) are highly homologues to MC synthetase genes.

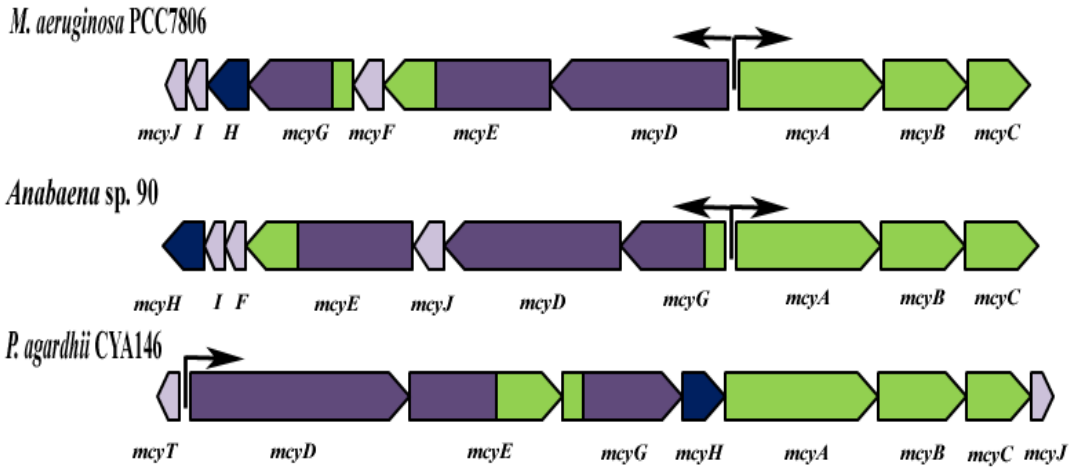


Fig.1.6. Organization of *mcy* gene clusters. The arrows show promoter regions. Colors: green, NRPS regions; violet, PKS regions; dark blue and mauve are other genes including tailoring genes and ABC transporters.

1.2.2. Cylindrospermopsin

1.2.2.1. Chemical structure

CYN is a highly water-soluble alkaloid, with a relative low molecular weight of 415 Da. CYN bears a tricyclic carbon skeleton, linked to a functional guanidine moiety attached to a hydroxymethyluracil, and a polar sulphate group. The structure of CYN was determined spectroscopically by Ohtani et al. (1992). Two natural CYN analogues involving C7 has been reported: the epimer, 7-epi CYN (Banker et al., 2000) and deoxy-CYN (Norris et al., 1999) lacking the hydroxyl group (Fig.1.8).

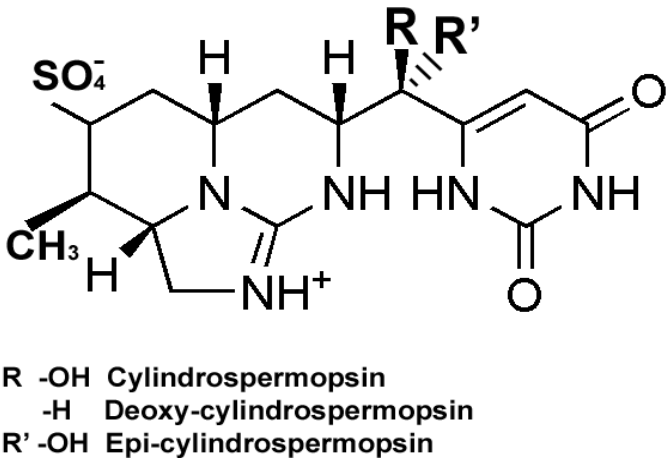


Fig.1.7. Chemical structure of cylindrospermopsin and its analogues.

An important aspect of CYN when considering its hazard is its significant stability, being resistant to widely different pH, temperature, sunlight (Chiswell et al., 1999) and to biological degradation (Wörmer et al., 2008).

1.2.2.2. Cylindrospermopsin toxicity

CYN toxicity was discovered upon the investigations (Ohtani et al., 1992) on the responsible compound of a poisoning outbreak in Palm Island (Queensland, Australia) in 1979, when 148 individuals fell sick after consumption of water from a pond containing toxic *C. raciborskii* (Griffiths and Saker, 2003). CYN is a protein synthesis inhibitor, affecting several cellular types of animals, man included. Due to this fact, it has been incorporated in the group of cytotoxins; but, since liver is the main target organ affected, it is also considered as hepatotoxin. Other human organs, as kidney (Falconer et al., 1999) and lung (Oliveira et al., 2012), can be damaged.

The toxicity mechanisms seem to be diverse, the following among them: reduced glutathione depletion (Runnegar et al., 1995c), reactive oxygen species (ROS) generation (Gutierrez-Praena et al., 2011 and 2012), inhibition of protein synthesis (Froscio et al., 2003), DNA damage (Zegura et al., 2011), genotoxic effects (Bazin et al., 2010 and 2012). Curiously, the main detoxification human system, Cytochrome-P450,

has been also implicated in CYN toxicity, due to the derived metabolites generated in its oxidation (Runnegar et al., 1995c; Humpage et al., 2005).

CYN has been detected in many ecosystems around the world, and it has become an important health human hazard in the last decade. Besides, CYN has been associated to livestock poisonings, causing also economical losses in cattle industry (Thomas et al., 1998; Saker et al., 1999). Plants are also affected by CYN (Metcalf et al., 2004; Beyer et al., 2009; Jambrik et al., 2010; Silva and Vasconcelos, 2010; Prieto et al., 2011; Kittler et al., 2012), representing a serious potential harm in agriculture and agroindustry. In principle, CYN and its analogues, epi-CYN and deoxy-CYN, were considered as tropical or subtropical toxins but they have been detected from temperate (Quesada et al., 2006; Fastner et al., 2007,) to boreal regions (Spoof et al., 2006). Its wide distribution and its high toxicity have made CYN and its congeners (epi-CYN and deoxy-CYN) compounds of great interest in water research and water management. A proof of such interest is the yearly increasing number of publications on these compounds (Fig.1.9).

Remarkably, the presence of CYN in Mediterranean countries has been mainly associated to *A. ovalisporum*. In Spain, cylindrospermopsin was firstly identified in 2006, in Arcos reservoir after a massive bloom episode of *A. ovalisporum* (Quesada et al., 2006). CYN was later reported in other places, also linked with the presence of *A.ovalisporum* (Wörmer et al., 2008, Barón-Sola et al., 2012). So far, CYN analyses are not mandatory in Spain, although a maximum limit of $1 \mu g.L^{-1}$ for drinking water is recommended.

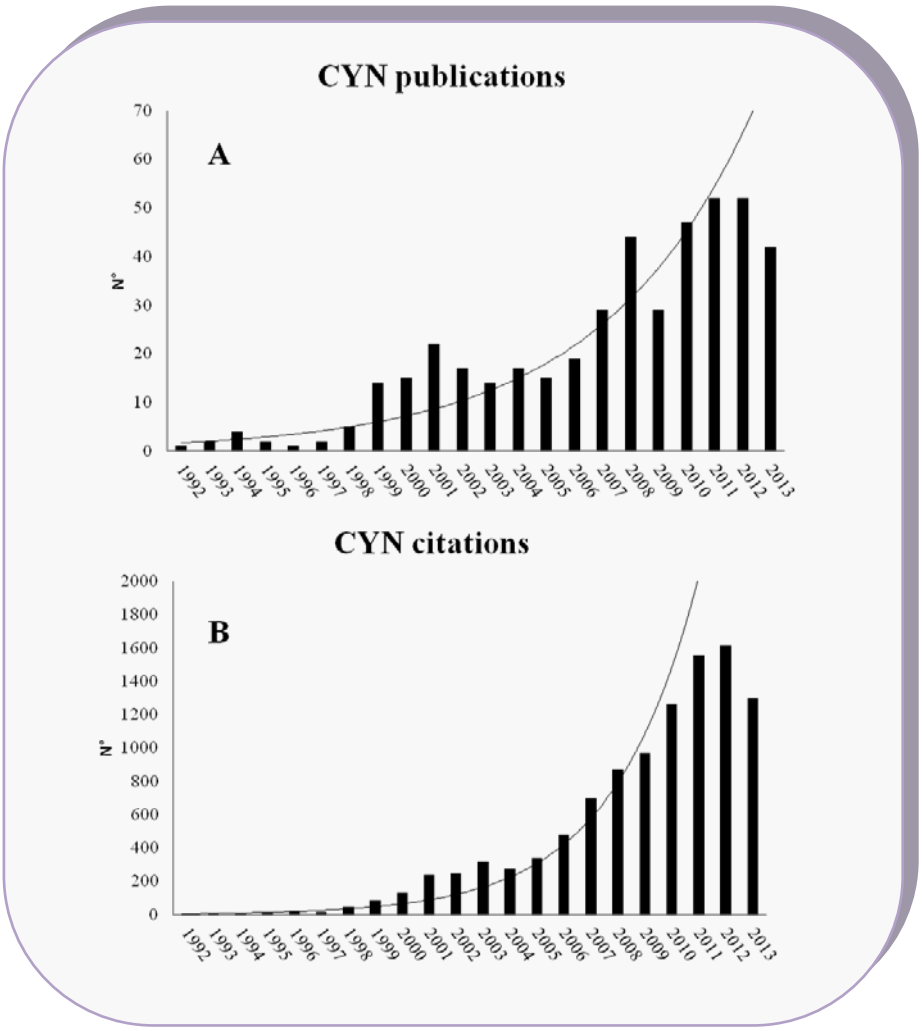


Fig.1.8. Cylindrospermopsin reports. A) Yearly published items. B) Yearly citations. (Source: Web of Knowledge[SM]). 2013 data were reported on October 2013.

The techniques used to detect CYN and/or evaluate its toxicity are in general, similar to those indicated for MCs: ELISA, HPLC, HPLC-MS, MALDI-TOF MS, NMR, bioassays and progressively more animal cell lines.

1.2.2.3. Genetics and synthesis

CYN is synthesized by several cyanobacterial species belonging to different genera such as *Cylindrospermopsis raciborskii* (Hawkins et al., 1985), *Umezakia natans* (Harada et al., 1994), *Aphanizomenon ovalisporum* (Banker et al., 1997), *Raphidiopsis curvata* (Li et al., 2001), *Anabaena bergii* (Schembri et al., 2001), *Anabaena lapponica* (Spoof et al., 2006), *Aphanizomenon flos-aquae* (Preussel et al., 2006), *Lyngbia wollei* (Seifert et al., 2007), *Oscillatoria* sp. (Mazmouz et al., 2010), *Raphidiopsis*

mediterranea (McGregor et al., 2011) and *Aphanizomenon gracile* (Kokocinski et al., 2013).

It seems significant that only *Nostocaceae* and *Oscillatoriaceae* are represented as CYN producers. Among the most representative CYN-producing species are *C. raciborskii* and *A. ovalisporum*, taking into account their extense distribution, elevated CYN production, and involvement in most CYN toxicity episodes.

Some gene regions are thought to be involved in CYN synthesis. The first report on putative CYN-synthesis responsible genes was by Shalev-Alon (2002), working with *A. ovalisporum*; hence, the name of *aoa* genes. Later, analogous genes, *cyr* (Fig.1.10), were found in *C. raciborskii* (Mihali et al., 2008), *Oscillatoria* sp. PCC 6506 (Mazmouz et al., 2010) and *Raphidiopsis curvata* (Jiang et al., 2012b). All clusters of genes *aoa* and *cyr* described show high sequence similarity; but, in the majority of them rearrangements in gene order are observed (Fig.1.10). In this respect, different phylogenetic analyses comparing DNA sequences of *cyr/aoa* clusters have suggested horizontal gene transfer of these genetic regions (Kellmann et al., 2006; Mazmouz et al., 2010; Stuken and Jakobsen, 2010; Jiang et al., 2012b).

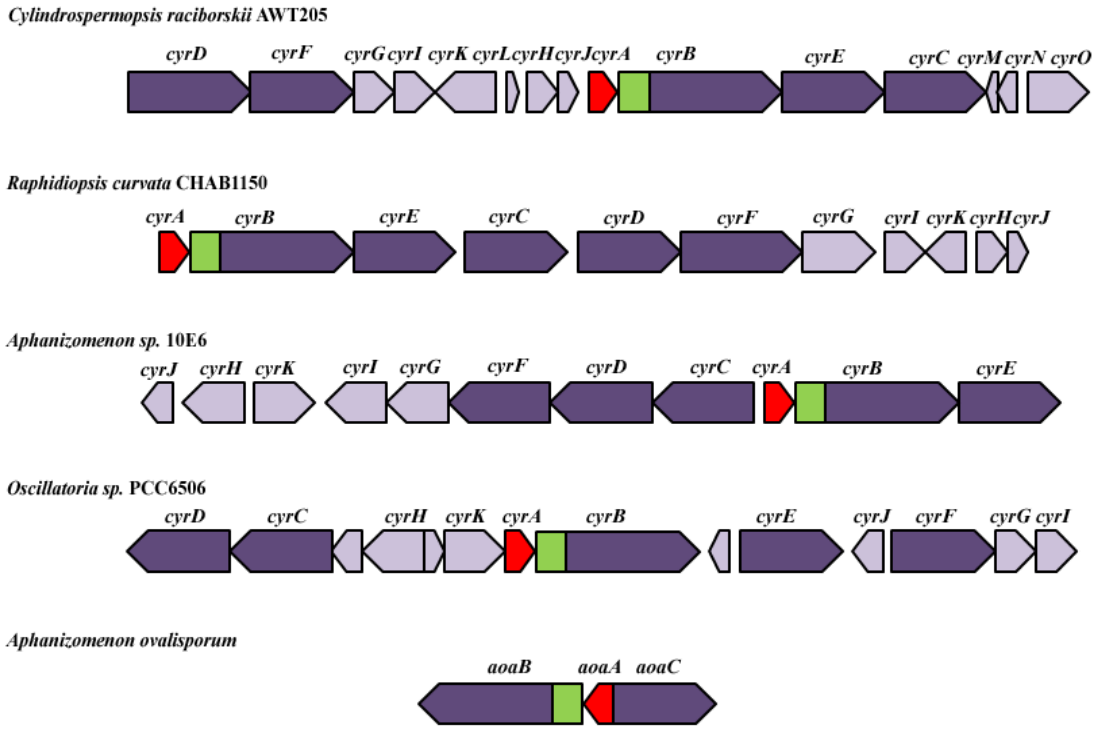


Fig.1.9. Organization of *aoa/cyr* gene clusters. Colors: red, amidinotransferase; green, NRPS regions; violet, PKS regions; mauve, other genes.

There is reasonable evidence for attributing to *aoa* and *cyr* genes an essential role in CYN synthesis. For example, *aoa*/*cyr* genes are solely present in CYN^+ cyanobacteria with one exception reported (Ballot et al., 2011) ; but to now, an experimental evidence to unequivocally attribute a direct implication of *aoa* and *cyr* in CYN synthesis is missing, having failed all the numerous attempts to obtain a knocked out mutant in any of the *aoa/cyr* genes.

As the *mcy* genes, *aoa* and *cyr* contain DNA sequences putatively codifying NRPS and PKS multimodular enzymes (Kellmann et al., 2008; Mihali et al., 2008). Further tailoring activities would contribute to synthesize the final molecule (Mihali et al., 2008; Mazmouz et al., 2011). The proposed pathway for CYN biosynthesis is depicted in Fig.1.11.

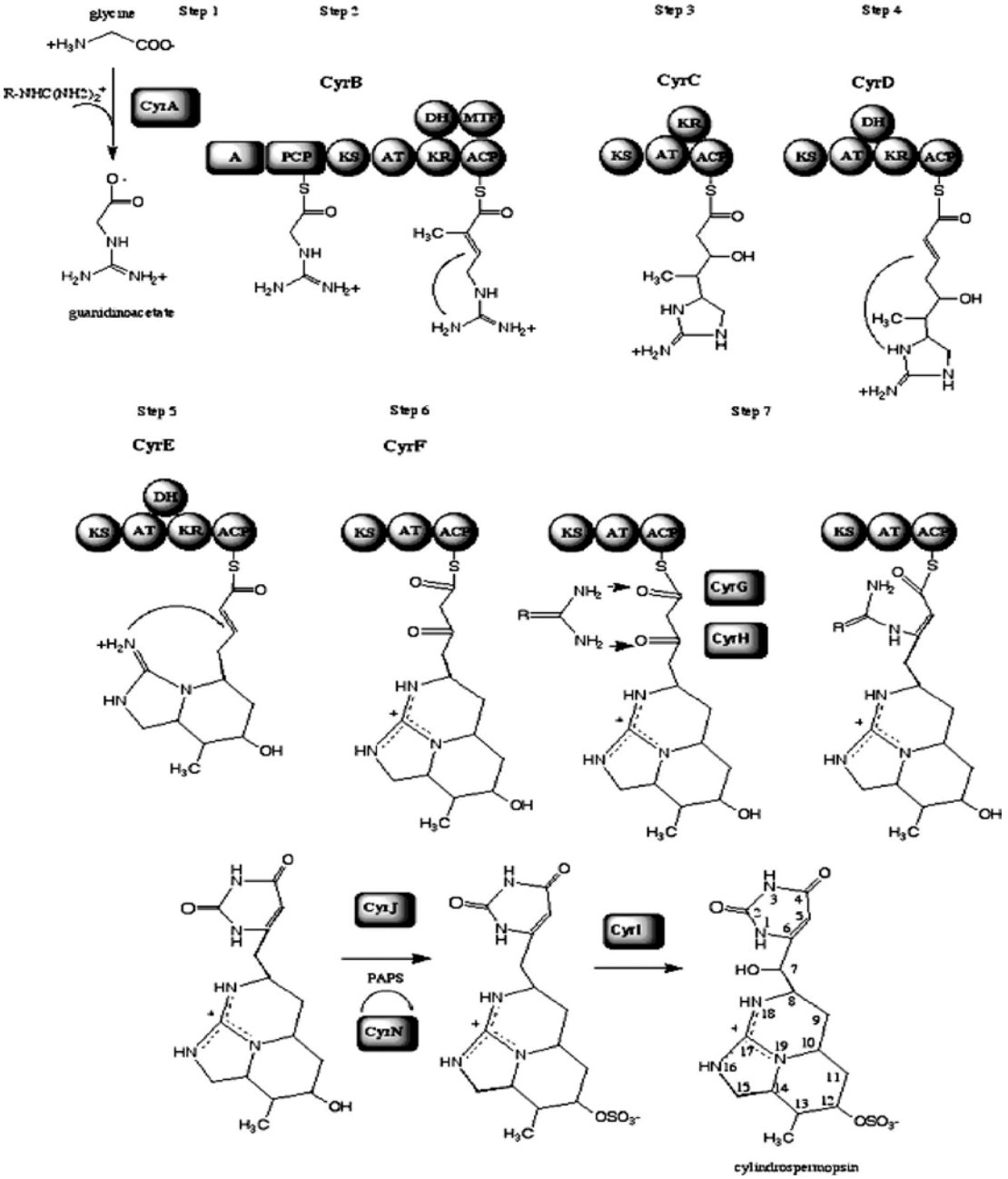


Fig.1.10. Proposed CYN biosynthesis pathway (Mihali et al., 2008).

Molecular studies revealed a transcriptional factor involved in the regulation of CYN synthesis in *Aphanizomenon ovalisporum* ILC-146 strain. An AbrB like protein was reported to bind to a promoter region situated between *aoaA* and *aoaC* genes. Two transcriptional star points were reported in the same region (Shalev-Malul et al., 2008). The absence of combined nitrogen and under light stress conditions alters the transcription activities of both *aoaA* and *aoaC* genes (Shalev-Malul et al., 2008). Moreover, CYN amount did not keep correlation with transcript abundance suggesting

other levels of regulation in toxin production. Multiple transcriptional start points have been also reported in other cyanobacterial genes such as *mcyA* and *mcyD* genes involved in MCs synthesis (Kaebernick et al., 2002).

In spite of the large genetic data compiled about CYN synthesis, the studies regarding the related enzymes are very scarce. So far, only enzymes participating in the supposed first and last steps of CYN biosynthesis pathway have been studied. The amidino transferase of *C. raciborski* AWT205, responsible for the first step of the proposed CYN synthesis, was obtained by cloning and overexpressing the gene *aoaA*, and further analyzed biochemically (Muenchhoff et al., 2010; Muenchhoff et al., 2012). The CyrA protein was the first cyanobacteria AMDT characterized, and exhibited distinct features from previously described AMDTs. It showed narrow substrate specificity, being arginine and glycine being the unique donor and acceptor respectively, and the activity followed a mixed ping-pong sequential enzymatic mechanism. With respect to the last step of CYN synthesis pathway, Mazmouz et al. (2011) obtained the protein codified by *cyrI*, CyrI, which acts as a 2-Oxoglutarate-Dependent Iron Oxygenase. There is a stereoselectivity for this enzyme to hydroxylate 7-deoxy-CYN producing either 7-epi-CYN or CYN.

The state of the art of cyanotoxicity just presented reveals an overall paucity of basic knowledge. An ample research on different fields is still needed to get to control cyanotoxicity in a rational manner. That research should be addressed to diverse basic aspects, the following among them: finding of new cyanotoxins as well as methodologies to analyze them accurately and to identify toxinogenic cyanobacteria; understanding as much as possible the synthesis of the diverse types of toxins, and the regulation of it; improvement and innovation of methodology to assay and evaluate cyanotoxicity; and understanding and evaluation of the relationship between cyanobacteria and other aquatic organisms.

2. OBJECTIVES

OBJECTIVES

As referred, the understanding of cyanotoxicity demands a multidisciplinary research and different experimental approaches. This work has focused in some of the demanding research fields: detection of cyanobacterial toxinogenic blooms, understanding of toxin synthesis and regulation, and exploration of new cyanotoxins. The specific objectives were:

1. To develop an assay for simultaneous detection of microcystin and cylindrospermopsin-producing cyanobacteria in environmental samples with mixed cyanobacteria populations.

2. To characterize the amidinotransferase from *A. ovalisporum* involved in the first step of cylindrospermopsin synthesis pathway.

3. To advance in understanding the regulation of CYN synthesis in *A. ovalisporum*, with special focus in transcriptional level.

4. To evaluate the accumulation of guanidinoacetate in different cyanobacteria, and to infer if this compound could be taken as a cyanotoxin.

5. To explore if the substrates of the characterized cyanobacteria AMDT affect CYN production in *A. ovalisporum.*

OBJETIVOS

El control sobre de la cianotoxicidad requiere un conocimiento profundo de numerosos aspectos, sólo posible mediante un abordaje multidisciplinar. Esta investigación se ha centrado en la detección de blooms con cianobacterias potencialmente tóxicas, regulación de la producción de toxinas, así como en la exploración de nuevas cianotoxinas. Los objetivos específicos fueron:

1. Desarrollar una metodología molecular para la detección simultánea de cianobacterias potencialmente productoras de MCs y CLP en muestras de campo con poblaciones heterogéneas de cianobacterias.

2. Caracterizar la amidinotransferasa de *Aphanizomenon ovalisporum* implicada en la ruta de síntesis de CLP.

3. Avanzar en el esclarecimiento de la regulación de la síntesis de CLP en *A. ovalisporum* a nivel transcripcional.

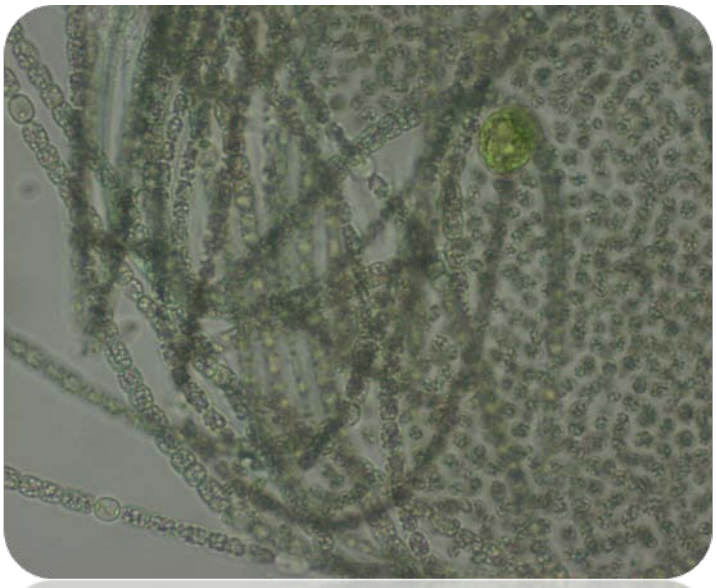
4. Evaluar la acumulación de guanidinoacetato en diferentes cianobacterias e inferir si este compuesto podría ser considerado como una cianotoxina.

5. Explorar en *Aphanizomenon ovalisporum* el efecto de los sustratos de la amidinotransferasa cianobacteriana implicada en cianobacterias en la producción de CLP.

3. RESULTS

3.1. CHAPTER I

Simultaneous detection of potentially producing cylindrospermopsin and microcystin cyanobacteria



Detection of potentially producing cylindrospermopsin and microcystin strains in mixed populations of cyanobacteria by simultaneous amplification of cylindrospermopsin and microcystin gene regions

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3.1.1. Abstract

Cyanobacterial blooms are frequently formed by heterogeneous populations of toxin-producing and non-producing strains. Microcystins (MC) and cylindrospermopsin (CYN) are the most representative cyanobacterial toxins. We have developed a multiplex PCR assay that allows simultaneous detection of MC^+ and/or CYN^+ strains in mixed populations of cyanobacteria. Various primer sets were designed using *mcy* and *aoa* gene sequences related with MC and CYN synthesis respectively, to amplify by multiplex PCR *aoa* and *mcy* sequences. Purified DNA, cultured cell mixtures and field samples with MC and CYN producing strains were used as DNA template. The results show: i) the expected amplicons were only observed with toxic strains; ii) cells were suitable as a source of purified DNA for the multiplex PCR; iii) the assay could detect simultaneously 3 *aoa* and 3 *mcy* gene regions with mixed CYN^+ and MC^+ cyanobacteria cells. Our data show that the method could be applied to environmental samples, allowing a rapid and economical test to easily detect simultaneously the presence of CYN^+ and MC^+ cyanobacteria in sestonic fractions of water samples.

Keywords: cylindrospermopsin, microcystin, *aoa* and *mcy* genes, multiplex PCR, cyanobacteria, environmental samples

3.1.2. Introduction

Planktonic cyanobacteria frequently overgrow under favorable conditions, forming blooms in different water bodies that cause different water quality concerns. Some of these organisms are capable of producing toxins, representing a serious health hazard

for animals and humans (Codd et al., 1999; Falconer, 1999; Carmichael, 2001; Van Apeldoorn et al., 2007).

The majority of cyanotoxin poisoning reports have been directly linked to two toxin types, cylindrospermopsin (CYN) and microcystins (MCs) (Falconer, 2005). Both toxins can be produced by various cyanobacteria genera around the world (Chorus and Bartram, 1999); consequently, monitoring of MCs and CYN in water bodies is increasing. There are studies on the concurrent presence of CYN and MCs in water (Bogialli et al., 2006; Brient et al., 2009; Oehrle et al., 2010), as well as on the coexistence of potentially CYN and MC-producing strains (Vasas et al., 2004; Blahova et al., 2008; Kokocinski et al., 2008).

Cylindrospermopsin is a widespread (Falconer and Humpage, 2005; Quesada et al., 2006; Spoof et al., 2006; Fastner et al., 2007) and stable (Chiswell et al., 1999; Wörmer et al., 2008) alkaloid. Its toxicity effects are diverse, a role in protein synthesis inhibition having been proposed (Froscio et al., 2003).Two CYN analog molecules have been reported: 7- epicylindrospermopsin, with similar toxicity to CYN (Banker et al., 2000), and deoxycylindrospermopsin, whose toxicity are well recognized by some authors (Looper et al., 2005; Neumann et al., 2007) but was questioned by others (Norris et al., 1999).

Several cyanobacteria species can produce CYN**,** the most representative being *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum*, causing most CYN occurrences up to now (Hawkins et al., 1985; Shaw et al., 1998; Briand et al., 2002; Blahova et al., 2008; Messineo et al., 2008). There is sound experimental evidence supporting the implication of a group of genes in CYN synthesis. In *A. ovalisporum* some of those genes, initially named *aoa*, (Shalev-Alon et al., 2001; Kellman et al., 2006) were clearly related with CYN production. The *aoa*A gene would encode an amidinotransferase (AMDT), *aoa*B a non-ribosomal peptide synthetase and polyketide synthase (NRPS/PKS) complex, and *aoa*C a PKS. Later, another CYN cluster gene (*cyr*) was identified in *C. raciborskii* (Mihali et al., 2008), including *aoaA-C* orthologs plus other genes. The involvement of the *cyrA* gene in CYN synthesis has been confirmed by overexpressing it and characterizing the codified protein as a novel AMDT (Muenchhoff et al., 2010). Recently, similar gene clusters have been described in *Aphanizomenon* sp. 10E6 (Stüken and Jakobsen, 2010) and *Oscillatoria* PCC6506 (Mazmouz et al., 2010). Besides, studies using PKS and NRPS gene determinants for

the specific identification of CYN-producing strains (Schembri et al., 2001; Fergusson and Saint, 2003) showed that only CYN^+ strains amplified those gene regions; and Rasmussen et al. (2008) reported the expression of *aoa* genes by real-time PCR just with CYN^+ strains.

MCs are the most common cyanobacterial toxins in water bodies, and to date more than 90 MC variants have been reported (del Campo and Ouahid, 2010). They are cyclic heptapeptides which toxicity is mainly based on the inhibition of protein phosphatases of types 1 and 2A (Mackintosh et al., 1990; Runnegar et al., 1995a and 1995b). The effects of MCs occur in various organs and tissues, but the best documented are on liver cells.

The gene system involved in MC synthesis has been thoroughly studied in several genera, *Microcystis* being the most representative. MCs are synthesized by the enzyme complex microcystin synthetase (MS), codified by *mcy* genes, that includes NRPS and PKS activities as well as other tailoring genes (Nishizawa et al., 1999; Tillet et al., 2000).

The control of cyanotoxicity is of worldwide interest, but it is not an easy task. Morphological identification of cyanobacteria cannot be used to identify toxin-producing strains; therefore, cyanotoxicity monitoring is based on cyanotoxin analysis in waters for direct consumption or recreational use. The methodologies and/or equipment to analyse cyanotoxins are relatively complex, expensive, require high concentration of cells or toxins, and the results can take a long time to become available. On the other hand, the appearance and dynamics of toxic blooms are unpredictable (Whitton, 2000), large changes in cyanotoxins having been observed in very short time periods, even 2-3 days. Seasonal studies show that cyanobacteria populations in different water sources are frequently formed by heterogeneous mixtures of toxin-producing and non-producing strains (Willen and Mattsson, 1995; Wood et al., 2006). Thus, early detection of toxin-producing cyanobacteria in the initial stages of bloom formation could alert agencies in charge of water surveillance on possible cyanotoxicity events.

Important advances have been made to molecularly identify potentially toxin-producing cyanobacteria. Multiplex PCR assays have been developed to amplify simultaneously several gene sequences involved in MC or CYN synthesis.

However, these assays were used to detect separately potentially MC^+ (Ouahid et al., 2005; Ouahid and del Campo, 2009; Valerio et al., 2010) or CYN^+ (Schembri et al., 2001; Fergusson and Saint, 2003; Kellman et al., 2006) cyanobacteria. We describe here the development of a multiplex PCR assay to type simultaneously potentially CYN^+ and MC^+ cyanobacteria. For such purpose, different specific sequences of *mcy* and *aoa* genes involved in MC and CYN synthesis, respectively, have been amplified simultaneously. To simplify the method, cells of heterogeneous cyanobacterial mixtures from cultures and environmental samples were utilized as DNA template.

3.1.3. Materials and methods

3.1.3.1. Biological material and culturing conditions

CYN^+ and MC^+ as well as CYN^- and MC^- cyanobacteria strains from different sources were used (Table 3.1.1). The UAM-MAO (MAO) and UAM-KMF (KMF) strains were isolated in our laboratory, MAO from a Spanish and KMF from a Moroccan reservoir. The UTEX strains were obtained from the University of Texas collection. The strains *C. raciborskii* VCC^+ and VCC^- as well as *A. ovalisporum* VAC^+ were kindly provided by Dr. Vasconcelos from the University of Porto, Portugal.

Table 3.1.1. Cyanobacterial strains used

Species	Strain	CYN[a]	MC[a]
Microcystis aeruginosa	**UTEX 2666**	-	+
Microcystis aeruginosa	**UTEX 2385**	-	+
Microcystis aeruginosa	**UTEX 2670**	-	+
Microcystis flos-aquae	**UAM KMF**	-	+
Aphanizomenon ovalisporum	**UAM MAO**	+	-
Aphanizomenon ovalisporum	**VAC^+**	+	-
Cylindrospermopsis raciborskii	**VCC^+**	+	-
Cylindrospermopsis raciborskii	**VCC^-**	-	-

[a] Toxin analyses were performed by HPLC-PDA

Environmental samples (Table 3.1.2) were obtained from two lakes: ALB from La Albufera (Valencia province, Spain), and TOR from El Toro (El Chaco province, Argentina). ALB was provided by Dr. Susana Romo (University of Valencia, Spain) and TOR by Drs. Juan Oteiza and Darío Andrinolo (University of La Plata, Argentina).

Table 3.1.2. Environmental samples used

Field sample	Potentially toxinogenic strain	Origin	Toxin[a]
Albufera (ALB)	***M. aeruginosa***	**Valencia, Spain**	**MC-YR,-LR,-FR**
Toro (TOR)	***C. raciborskii***	**El Chaco, Argentina**	**CYN**

[a] Toxin analyses were performed by HPLC-PDA

Cyanobacteria identification was carried out according to Komárek and Anagnostidis (1999). All strains were cultured in J medium (Corbett and Parker, 1976) without agitation, under continuous fluorescence light (40 µmol m^{-2} s^{-1} PAR), at 25 ºC. The quantification of cyanobacteria for multiplex PCR assays was carried out using a hemocytometer chamber under an Olympus BH-2 RFCA microscope equipped with a Leica DC300F digital camera.

3.1.3.2. Toxin analyses

Cyanotoxin analyses were performed with cyanobacteria extracts by high performance liquid chromatography with photodiode array (HPLC-PDA, Agilent 1200 equipment). MCs and CYN were extracted from lyophilized biomass with 75% methanol in water, and 5% aqueous formic acid as extraction solvents, respectively. The HPLC methods for MC and CYN were those of Lawton et al. (1994) and Törökne et al. (2004), respectively. MCs and CYN standards were purchased from Abraxis. Toxin identification was carried out by UV absorption spectra and HPLC retention times.

3.1.3.3. Primer design and PCR assays

In order to detect CYN and MC-producing cyanobacteria specific primers were used to amplify different gene fragments, with a consensus sequence for a characteristic domain plus flanking regions (Table 3.1.3). All the primers used for CYN^+ detection were newly designed. The CatF1/ CatR1 primer set allowed to amplify an amidinotransferase (AMDT) sequence included in the *aoaA* gene; the Cpb2/CpbR2 set permitted to amplify a sequence from the hybrid *aoaB* gene that codifies for NRPS/PKS activities; and the

CkcF3/CkcR3 set would allow amplifying a PKS sequence from the *aoaC* gene. The three primer sets to amplify *mcy* sequences, codifying for NRPS and PKS regions (PSCF3/ PSCR3¸ PKEF1/ PKER1, PKGF1/ PKGR1) were those previously designed in our lab (Ouahid et al., 2005; Ouahid and del Campo, 2009). Finally, the *cpcBA*-IGS primers (Neilan et al., 1995) were used to amplify phycocyanin gene regions (*cpc*), as a control for the presence of cyanobacteria.

Table 3.1.3. Primers used for multiplex PCR assay

Primer	Primer sequence (5'-3')	Target sequence	Product size (bp)	Reference
CatF1	agatggtgcttattttgaac	*aoaA*	881	This study
CatR1	tcttcacagatgaccttctt			
CpbF2	caccattggctatgtagaagct	*aoaB*	550	This study
CpbR2	tattggctgtgaaagagaggtc			
CkcF3	aatgatcgaaaacagcagtcgg	*aoaC*	325	This study
CkcR3	tagaacaatcatcccacaacct			
PSCF3	ggtgtttaataaggagcaag	*mcyC*	648	Ouahid and del Campo, 2009
PSCR3	attgataatcagagcgtttt			
PKEF1	cgcaaacccgatttacag	*mcyE*	755	Ouahid et al. 2005
PKER1	cccctaccatcttcatcttc			
PKGF1	actctcaagttatcctccctc	*mcyG*	425	Ouahid et al. 2005
PKGR1	aatcgctaaaacgccacc			
Cpβ F	ggctgcttgtttacgcgaca	*cpcBA*-IGS	650	Neilan, 1995
Cpα R	ccagtaccaccagcaactaa			

PCR was carried out using purified DNA, cultured cells and field cyanobacteria biomass as previously described (Ouahid et al., 2005).

The PCR reaction mixture contained in 25 μL: 2.5 μL of PCR buffer 10X (Biotools, Spain), 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.1 mg mL^{-1} of BSA, and 1U of Taq DNA polymerase. The amount of each primer in a multiplex reaction depended on the combination of the primers set used. For CYN^+ detection, the amount of Cat, Cpb and Ckc primer set was 30, 25 and 15 pmol, respectively. For MC^+ typing, the PSC, PKG and PKE primer set amount was 20, 10 and 25 pmol respectively. The CPC primer set quantity was 30 pmol. The multiplex PCR assays were carried out with 50 ng of genomic DNA or 60,000 cells, except where indicated. PCR conditions were: initial

denaturation at 94 ºC for 7 min, followed by 40 cycles of denaturing at 94 ºC for 30 s, annealing at 54 ºC for 1 min, elongation at 72 ºC for 1 min, and final extension at 72 ºC during 10 min.

All experiments were carried out in a Techne model FTGENE2D thermocycler, and PCR products were visualized in a 1.5% (w/v) agarose gel stained with ethydium bromide or GelRed^{TM} Nucleic Acid Gel Stain.

3.1.4. Results

3.1.4.1. Detection of cylindrospermopsin-producing cyanobacteria by Multiplex PCR with cell suspensions

Three *aoa* regions (*aoaA*, *aoaB* and *aoaC*), and the phycocyanin (*cpcBA*-IGS) gene region as a positive control for cyanobacteria presence , were amplified simultaneously using cell suspensions of different CYN^+ and CYN^- strains (Table 3.1.1). Figure 3.1.1 shows the results of a multiplex PCR assay with CatF1/CatR1, CpbF2/CpbR2 , CkcF3/CkcR3 and CpβF/CpαR primers. Four amplicons were obtained with CYN^+ strains. One (650 bp) corresponded to *cpc*, and the rest showed the size of the expected *aoa* fragments: 881 bp (*aoaA*), 550 bp (*aoaB*) and 325 bp (*aoaC*). With the CYN^- strains only the *cpc* fragment was obtained. The *aoa* identity was confirmed by sequencing the amplified fragments.The sequences have being deposited in the GenBank data base, under the accession numbers JN003450-60 (supplementary material).

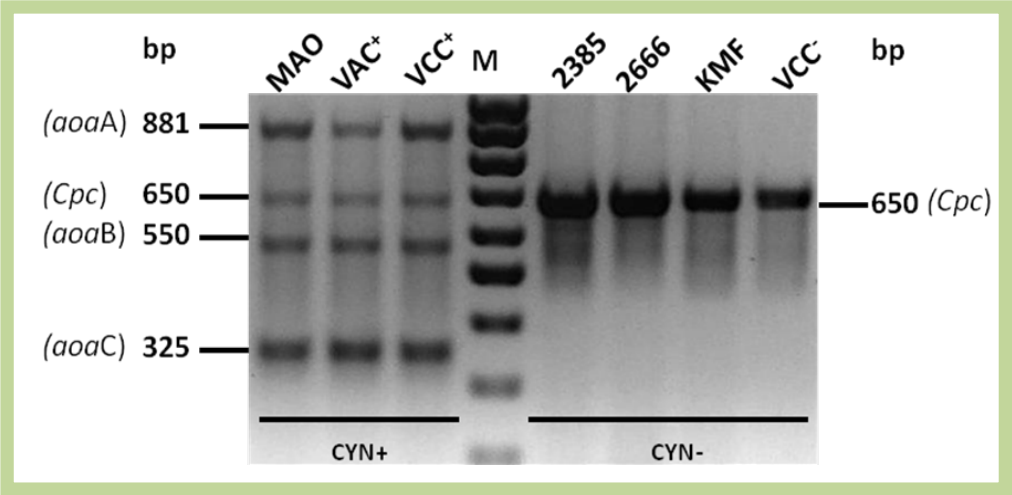

Fig.3.1.1. Amplification of *aoa* and *cpc* sequences by multiplex PCR with cells of CYN^+ and CYN^- strains as DNA template. Primers used: CatF1/CatR1 to amplify an AMT region (*aoaA*,881 bp); CpbF2/CpbR2 for a mixed NRPS/PKS region (*aoaB*, 550 bp); and CkcF3/CkcR3 for a PKS sequence (*aoaC*, 325 bp). The c*pcBA*-IGS sequence (650 bp) corresponds to a phycocyanin gene region amplified as a positive control for cyanobacteria DNA. M, DNA marker.

3.1.4.2. Multiplex PCR for simultaneous detection of CYN+ and MC+ cyanobacteria

To explore the compatibility of primers to amplify simultaneously *mcy* and *aoa* sequences in the same PCR reaction, different CYN^+ and MC^+ cyanobacteria (Table 3.1.1) were utilized. The *cpcBA*-IGS primer set was used as a positive control of cyanobacteria presence. The expected amplicons were only obtained in potentially toxin-producing strains (Fig. 3.1.2). The *aoaA* and *aoaC* gene regions (lanes 1-3) were amplified in the CYN^+ strains, and the *mcyE* and *mcyG* sequences in the MC^+ strains (lanes 4-7). Both CYN^+ and MC^+ cyanobacterial strains amplified the phycocyanin gene region.

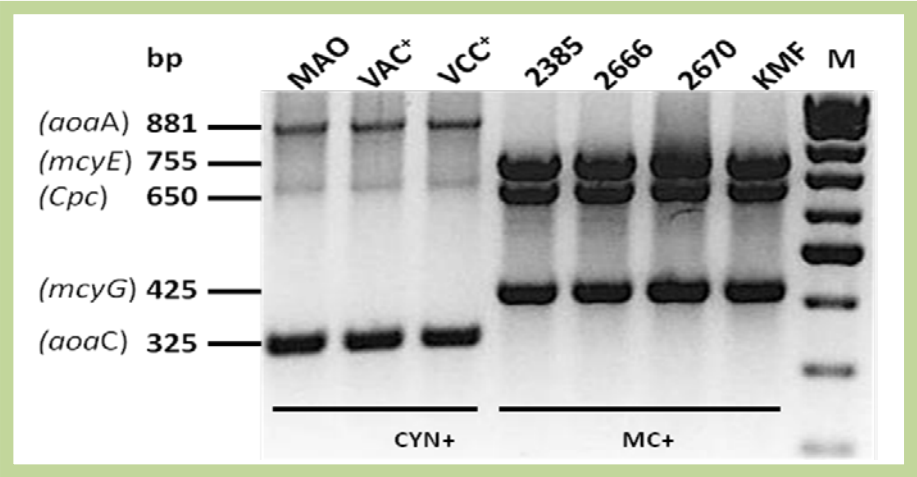

Fig.3.1.2. Amplification of *aoa* and *mcy* sequences with cells of CYN^+ and MC^+ strains as DNA source. Amplification of specific *aoaA* region using CatF1/CatR1 (881 bp), *aoaC* region using CkcF3/CkcR3 (325 bp), *mcy*E sequence with PKEF1/PKER1(755bp), and *mcyG* gene region using PKGF1/PKGR1 (425bp) . The *cpcBA*-IGS sequence (650 bp) used as a cyanobacterial control. M DNA marker.

The efficiency of whole cells as DNA template in the multiplex PCR was assessed by comparing gene amplification with cells and purified DNA. Figure 3.1.3 shows the results of three experiments using cell or DNA mixtures of two different strains, one MC^+ (*M. aeruginosa* strain UTEX2385) and one CYN^+ (*A. ovalisporum* UAM-MAO). In all cases, the same starting amount of cells was used, 30.000, that yielded ca. 25 ng purified DNA. The primer sets utilized were: in A, CatF1/CatR1, CpbF2/CpbR2 to amplify *aoa* sequences, and PSCF3/PSCR3 and PKGF1/PKGR1 to amplify *mcy* gene regions and 30, 20, 25 and 10 pmol respectively; in B, CatF1/CatR1, CpbF2/CpbR2 for *aoa* amplification, and PSCF3/PSCR3 for a *mcy* sequence; and in C, CatF1/CatR1 for *aoa* amplification, and PSCF3/PSCR3 and PKGF1/PKGR1 for *mcy* sequences. The expected amplicons were obtained with each mixture: with A, 2 *aoa* and 2 *mcy*; with B, 2 *aoa* and 1 *mcy*; and with C, 1 *aoa* + 2 *mcy*. No significant differences were observed between DNA and cells, suggesting that cells were as suitable as purified DNA for multiplex PCR assays.

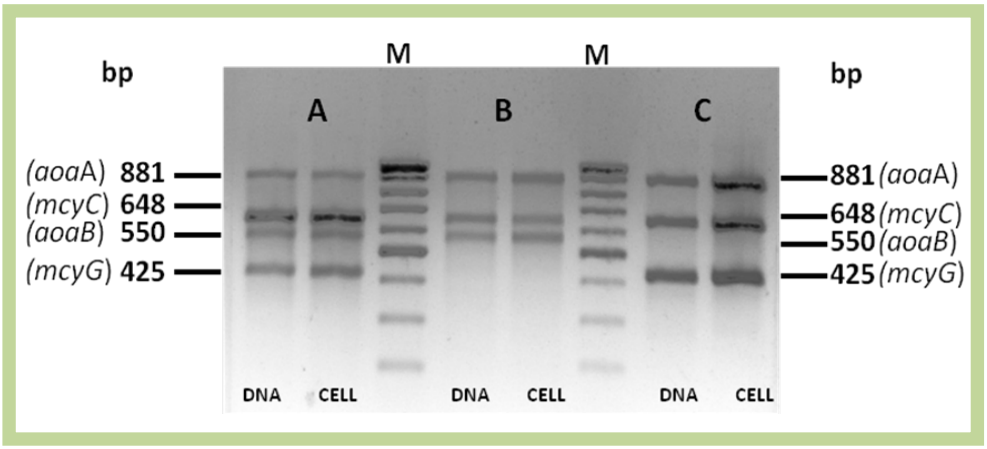

Fig.3.1.3. Multiplex of *aoa* and *mcy* sequences with different primer set combination comparing purified DNA and cell mixtures of a CYN^+ strain (*A. ovalisporum* UAM-MAO) and MC^+ strain (*M. aeruginosa* UTEX2385) as a template. **A,** Amplification of 4 sequences corresponding to *aoaA* (881 bp), *aoaB* (550 bp), *mcyC* (648 bp) and *mcyG* (425 bp) using CatF1/CatR1, CpbF2/CpbR2, PSCF3/PSCR3 and PKGF1/PKGR1 primers. **B,** Amplification of 3 sequences corresponding to *aoa*A (881 bp), *aoaB* (550 bp), *mcyC* (648 bp) using CatF1/CatR1, CpbF2/CpbR2, PSCF3/PSCR3 primer sets. **C,** Multiplex amplifying 3 gene regions *aoaA* (881 bp), *aoaB* (550 bp) and *mcyG* (425 bp) using CatF1/CatR1, CpbF2/CpbR2, and PKGF1/PKGR1 primers. M DNA marker.

We further applied the method using six primer sets, to amplify 3 *aoa* + 3 *mcy* sequences simultaneously. In this instance, also mixtures with purified DNA and whole cells of *C. raciborskii* (strain VCC^+) and the *M. aeruginosa* (strain UTEX2666) as a template were used. Again, all the expected amplicons were obtained (Fig. 3.1.4). But, different concentration of primers had to be utilized for an optimal amplification depending of the primer set combination. Thus, the concentrations of *aoa* primers, CatF1/CatR1, CpbF2/CpbR2, CkcF3/CkcR3 were 40, 30 and 20 pmol respectively; and the concentration of *mcy* primers PSCF3/PSCR3, PKEF1/PKER1 and PKGF1/PKGR1 was 25, 30 and 15 pmol, respectively.

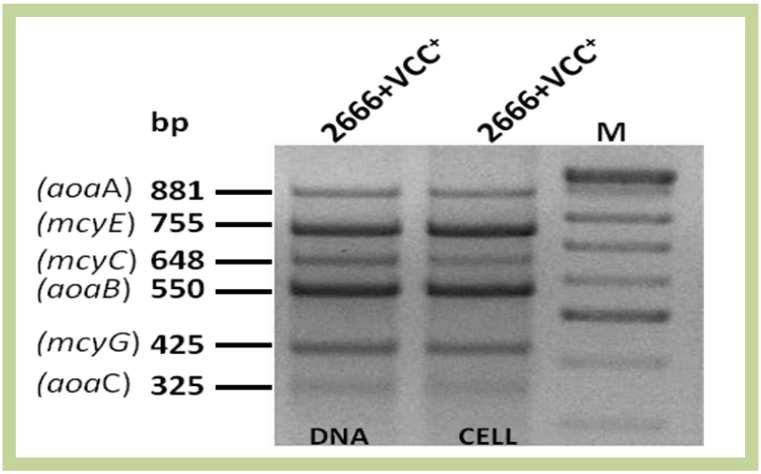

Fig.3.1.4. Amplification of 6 sequences (3 *aoa* and 3*mcy*) using purified DNA and cell mixtures of a CYN^+ strain (*C. raciborskii* VCC^+) and a MC^+ strain (*M. aeruginosa* UTEX2666) as DNA template. Amplification of *aoaA* (881 bp), *aoaB* (550 bp), *aoaC* (325 bp), *mcyC* (648 bp), *mcyE* (755 bp) and *mcyG* (425 bp) gene regions using CatF1/CatR1, CpbF2/CpbR2, CkcF3/CkcR3, PSCF3/PSCR3, PKEF1/PKER1 and PKGF1/PKGR1 primers set, respectively. M DNA marker

The nucleotide sequence of *aoa* fragments obtained with *A. ovalisporum* (Fig. 3.1.3) and *C. raciborskii* (Fig. 3.1.4) were almost identical (ca. 99% identity).

3.1.4.3. Detection of CYN^+ and MC^+ cyanobacteria in environmental samples

A further step to study the applicability of the multiplex PCR method was to check if environmental samples as a DNA source could be directly used. Phytoplankton samples from two different water bodies (Albufera de Valencia Lake, Spain; and Laguna El Toro from El Chaco, Argentina), containing potential toxin-producing cyanobacteria such as *C. raciborskii* (Toro) and *M. aeruginosa* (Albufera), were analyzed by HPLC to confirm the presence of CYN and/or MCs, respectively. We detected CYN in the Toro sample (Fig. 3.1.5) and several MCs in the Albufera sample (Fig. 3.1.6).

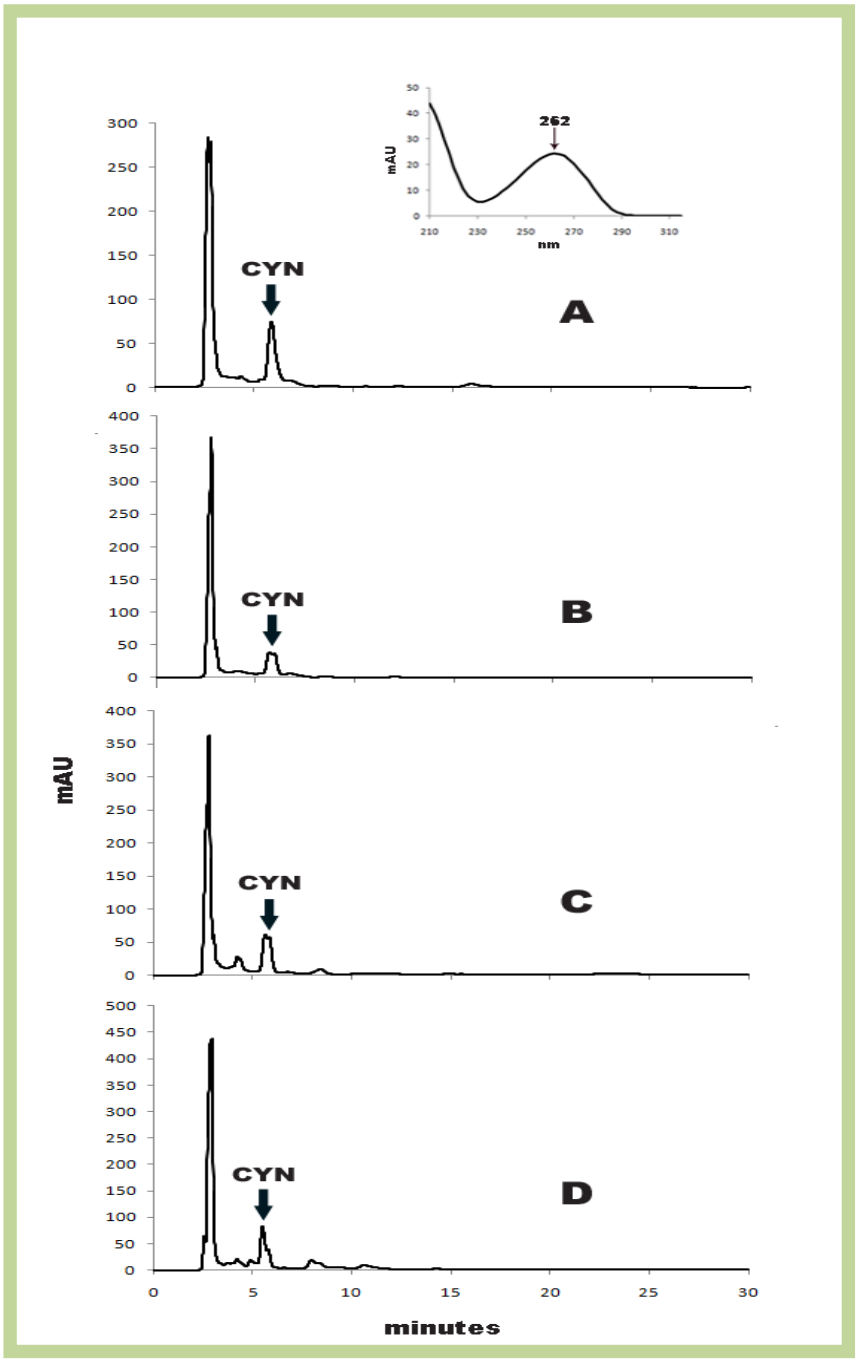

Fig.3.1.5. HPLC chromatograms of extracts from different CYN^+ cyanobacterial strains and one phytoplankton sample: **A,** *A. ovalisporum* UAM-MAO. **B,** *A. ovalisporum* VAC^+. **C,** *C. raciborskii* VCC^+. **D,** Toro reservoir, Argentina (TOR). Insert, detailed CYN UV spectrum.

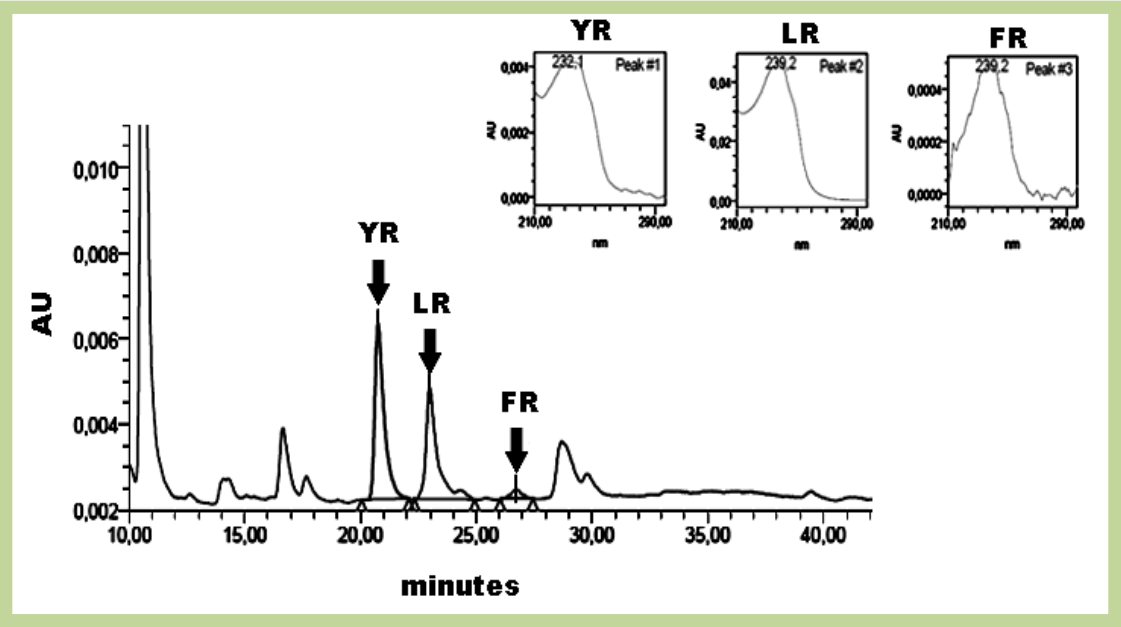

Fig.3.1.6. HPLC chromatogram of a phytoplankton sample from the Albufera lake, Spain (ALB). Insert, detailed UV spectra of MC-YR,-LR and-FR.

Then, we performed multiplex assays with CatF1/CatR (30 pmol), CkcF3/CkcR3 (15 pmol), PKEF1/PKER1(25 pmol) and PKGF1/PKGR1(10 pmol) primer sets. The two environmental samples were used separately and mixed. The results obtained appear in Figure 3.1.7. As expected, with the Albufera sample, containing *M. aeruginosa*, *mcyE* and *mcyG* regions were amplified; and with the Toro sample, containing *C. raciborskii*, *aoaA* and *aoaC* amplicons were obtained. Furthermore, the mixture of the Toro and Albufera samples showed the four predicted bands.

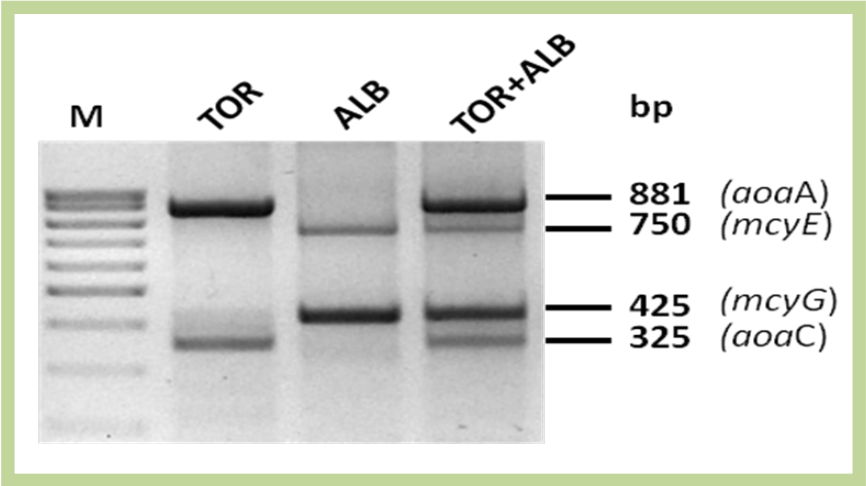

Fig.3.1.7. Amplification of *aoa* and *mcy* sequences using environmental samples as a template. The primer set mixture utilized was: CatF1/CatR1, CkcF3/CkcR3, PKEF1/PKER1 and PKGF1/PKGR1. First lane (TOR), shows amplification of *aoa*A (881 bp) and *aoa*B (550 bp) gene regions with a phytoplankton sample from Toro reservoir (Argentina) containing potentially CYN^+ *C. raciborskii*. Second lane (ALB): amplification of *mcy*E (755 bp) and *mcy*G (425 bp) using a field sample from Albufera lake (Spain) with potentially MC^+ *M. aeruginosa*. Third lane (TOR+ALB) amplification of *aoaA* (881 bp), *aoaC* (325 bp), *mcyE* (755 bp) and *mcyG* (425 bp) sequences using a mixture of Toro and Albufera samples as a template. M DNA marker.

3.1.5. Discussion

The aim of our work was to set up a simple and reliable method to easily detect simultaneously CYN^+ and MC^+ strains in phytoplankton samples with mixed cyanobacteria populations. We have developed a multiplex PCR assay to amplify simultaneously up to three *aoa* and three *mcy* gene regions putatively involved in CYN and MC synthesis, respectively (Fig. 3.1.4). The methodology represents a step forward in the use of multiplex PCR to identify cyanotoxin-producing cyanobacteria, which until now has been utilized to separately detect either CYN^+ (Schembri et al., 2001; Fergusson and Saint, 2003; Kellman et al., 2006) or MC^+ (Ouahid et al., 2005; Ouellete et al., 2006; Ouahid and del Campo 2009; Valerio et al., 2010) strains. Most of our results refer to *A. ovalisporum*, but considering the high identity of the *aoa* amplicons observed with *A. ovalisporum* and *C. raciborskii* (supplementary data) it is reasonable to think that the new methodology might be used to detect different CYN^+ species.

Several aspects appeared important for the success of the multiplex PCR. Firstly, the use of specific primers to amplify the desired *aoa* and *mcy* gene sequences, preventing the amplification of similar sequences unrelated to cyanotoxins. To detect *aoa* genes,

new primers were designed to allow the amplification of *aoaA-C* gene regions (Table 3.1.3), including sequences of putative key enzymes in CYN synthesis: AMDT, PKS and NRPS. Another key aspect was the optimization of the amplification of all the gene sequences. For that, the concentration of each primer should be changed depending on the combination of primers used (Figs. 3.1.3 and 3.1.4). This aspect was already considered in our laboratory when amplifying simultaneously *mcy* regions (Ouahid and del Campo, 2009). In spite of these cautions, frequently not all the expected amplicons showed similar intensity (Figs.3.1.4 and 3.1.5), and in some instances even some of the expected amplicons were not observed. Nevertheless, even with suboptimal primer concentration some of the expected *aoa* and *mcy* amplicons were always obtained. This fact is one of the reasons to emphasize that multiplex PCR should be considered as a more reliable methodology than simple PCR to type toxin-producing Cyanobacteria, where more false negatives often appear.

The new multiplex PCR was effective with cell cultures or phytoplankton biomass, a fact that undoubtedly simplifies and widen the utility of the assay. Recently, we reported the suitability of cells and field samples as DNA template in multiplex PCR to amplify up to six *mcy* genes using cells (Ouahid and del Campo, 2009). Other authors have detected CYN^+ strains by multiplex PCR of *aoa* sequences, but in the assays purified DNA was always used (Schembri et al., 2001; Fergusson and Saint, 2003; Kellman et al., 2006).

Although the presence of genetic toxin determinants does not mean necessarily toxin production (Neilan et al., 1999; Tillet et al., 2001), the multiplex developed represents a new methodology for a simple, rapid and sensitive diagnosis of potentially CYN and MC-producing cyanobacteria in phytoplankton. It could be used to alert agencies in charge of water surveillance on the possibility of cyanotoxicity events, and help them to be ready for eventual cyanotoxin decontamination.

We would like to remark that one of the CYN^+ environmental samples utilized was from Argentina (Toro, Chaco region), because as far as we know this is the first time that the presence of CYN has been reported in Argentinean waters. It would be worthwhile to extend CYN monitoring not only to the whole Argentinean territory, but to other Latin American countries.

Acknowledgments

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3.1.6. Complementary work

3.1.6.1. Suitability and sensitivity of whole cells as DNA template for multiplex PCR

In order to compare the suitability and sensitivity of DNA and cells as PCR template, different amounts of both were tested for *aoa* gene amplification. To start, one primer pair was used. 5 ng of DNA or only 500 cells were enough to obtain the expected amplicon, indicating that whole cells used as PCR template are an appropriate that affords a simplified method for *aoa* gene amplification by avoiding DNA purification steps. Later, the simultaneous amplification of two *aoa* gene sequences was also assayed. Figure 3.1.8 shows that both expected PCR products, 881 bp (*aoaA*) and 560 bp (*aoaB*) were obtained. On the other hand, the sensitivity did not change with respect to the experiments using a single pair of *aoa* primers.

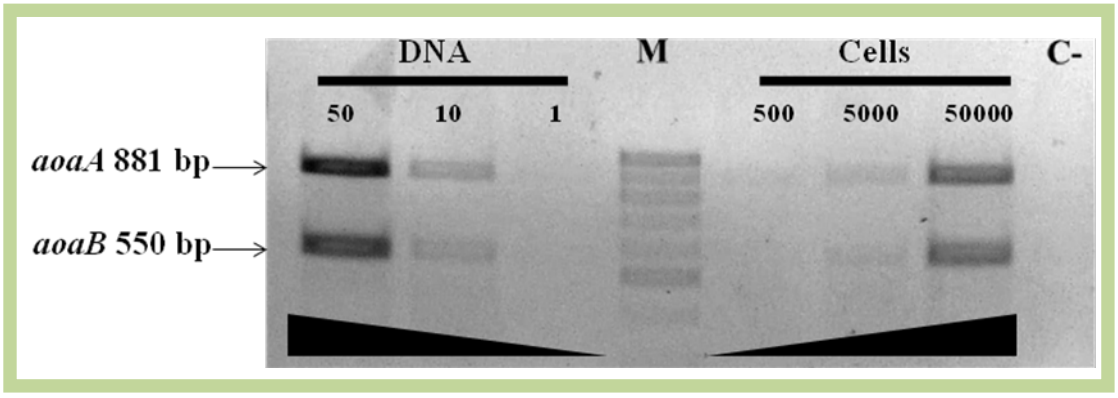


Fig.3.1.8. Comparison of the sensitivity of DNA and cells as template for multiplex PCR. Amplification of *aoaA* and *aoaB* gene sequences of CYN^+ *A. ovalisporum*. The primer set mixtures utilized were CatF1/CatR1 and CpbF2/CpbR2. C^-, negative control. M, molecular marker. Numbers show the amount of cells or DNA (ng) used.

The same primer sets, *aoaA* and *aoaB*, as well as *aoaC*,were assayed using CYN^+ and CYN^- strains to validate specificity and suitability of the method in other CYN^+ species. These initial experiments allowed concluding that several *aoa* sequences could be specifically amplified by multiplex PCR in CYN^+ strains. Then, in order to ensure the

assignment of CYN production to cyanobacteria other primer set (*aoaC*) was added to the multiplex PCR.

3.1.6.2. Interference of other cyanobacteria in multiplex PCR assay

To check if the multiplex methodology could be applied to field samples, we proceeded to test a possible interference of cyanobacteria of diverse type, considering that blooms are often formed by heterogenous cyanobacteria populations. Several mixtures of whole cells of filamentous and unicellular CYN^- strains with CYN^+ cyanobacteria were performed, and three *aoa* primer sets were used simultaneously (Fig. 3.1.9).

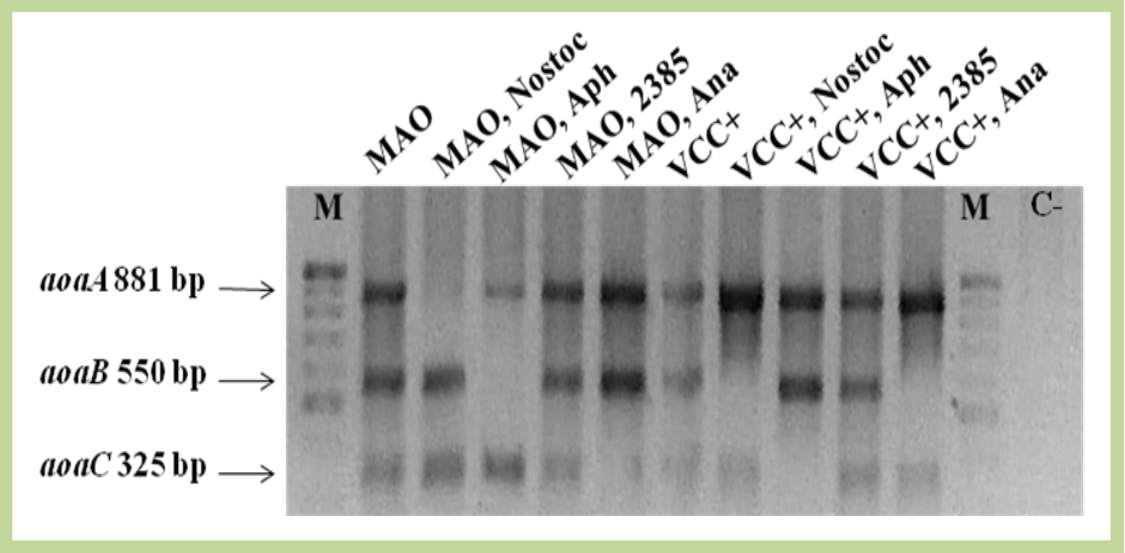


Fig.3.1.9. Multiplex PCR for *aoa* sequences amplification using cell biomass from different cyanobacteria mixtures. The primer set mixtures utilized were: CatF1/CatR1, CpbF2/CpbR2, CkcF3/CkcR3. CYN^+: *A. ovalisporum* UAM-MAO (MAO) and *C. raciborskii* (VCC^+); CYN^-**:** *Nostoc* sp, *A. aphanizomenoides* (Aph), *M. aeruginosa* UTEX2385 (2385) and *Anabaena flos-aquae* (Ana). Cell amount per reaction, 50000.

The results showed that, in effect, there was interference in the multiplex PCR reaction when mixtures of cyanobacteria were used (Fig.3.1.9). Nevertheless, despite the high variability on the amplification, at least two amplicons were obtained in every single sample used.

3.1.6.3. Application to field samples

The new multiplex was applied to environmental samples from two Spanish water bodies (Table 3.1.4), using directly sestonic biomass as DNA template. In these samples both CYN and MCs were found; thus, they should include CYN^+ and MC^+ strains. To simultaneously detect the two classes of cyanobacteria, *aoa* and *mcy* sequences were amplified.

Table 3.1.4. Environmental samples utilized for simultaneous amplification of *aoa* and *mcy* sequences.

Sample	Date	Waterbody	Cyanotoxin	Potentially toxic cyanobacteria
I	July 2005	PJCI	CYN/MC[a]	*A.ovalisporum/M.aeruginosa*
II	August 2005	PJCI	CYN/MC[a]	*A.ovalisporum/M.aeruginosa*
III	September 2005	PJCI	CYN/MC[a]	*A.ovalisporum/M.aeruginosa*
IV	August 2010	Alange	CYN/MC[b]	*A.ovalisporum/M.aeruginosa*
V	September 2010	Alange	CYN/MC[b]	*A.ovalisporum/M.aeruginosa*

[a] CYN and MC from Parque Juan Carlos I (PJCI) samples were detected by HPLC-PDA following methods described by Törökné et al. (2004) and Fastner et al. (2002), respectively.[b] CEDEX, 2010

As expected, all environmental samples containing *MC and* and CYN, with potentially producers of these toxins, amplified *mcy* and *aoa* determinants (Fig. 3.1.10). However, not all amplicons were detected in each sample tested, due to interferences, as described above. Therefore, the increasing of molecular determinants in multiplex PCR assays would prevent false negatives.

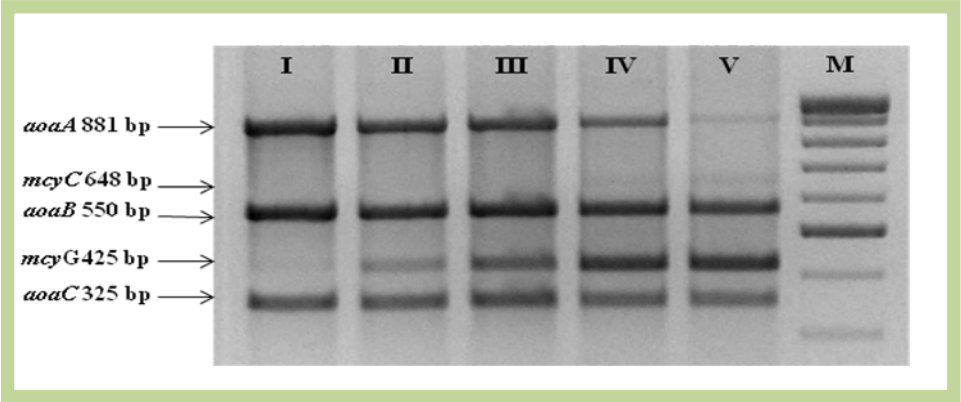


Fig.3.1.10. Amplification of *aoa* and *mcy* sequences using environmental samples as DNA template. The primer set mixtures used were: CatF1/CatR1, CpbF2/CpbR2, CkcF3/CkcR3, PSCF3/PSCR3 and PKGF1/PKGR1. The samples utilized are described in Table 3.1.5.

Taking together the methodology described here, based on multiplex PCR, allows the reliable detection of potentially CYN^+ and MC^+ cyanobacteria, using whole cells or environemtal samples as DNA template.

All multiplex PCR assays of this section were as described in materials and methods section in the published work of chapter I.

3.1.6.4. CYN analysis by LC-MS-MS

All extracts from CYN^+ strains used in this work were analyzed by both HPLC-PDA (see the published work of this Chapter) and mass spectrometry. CYN was extracted from lyophilized biomass according to Törökné et al. (2004), and LC-MS-MS assays were performed after Spoof et al. (2006). Some of the analysis data are gathered in Figure 3.1.11.

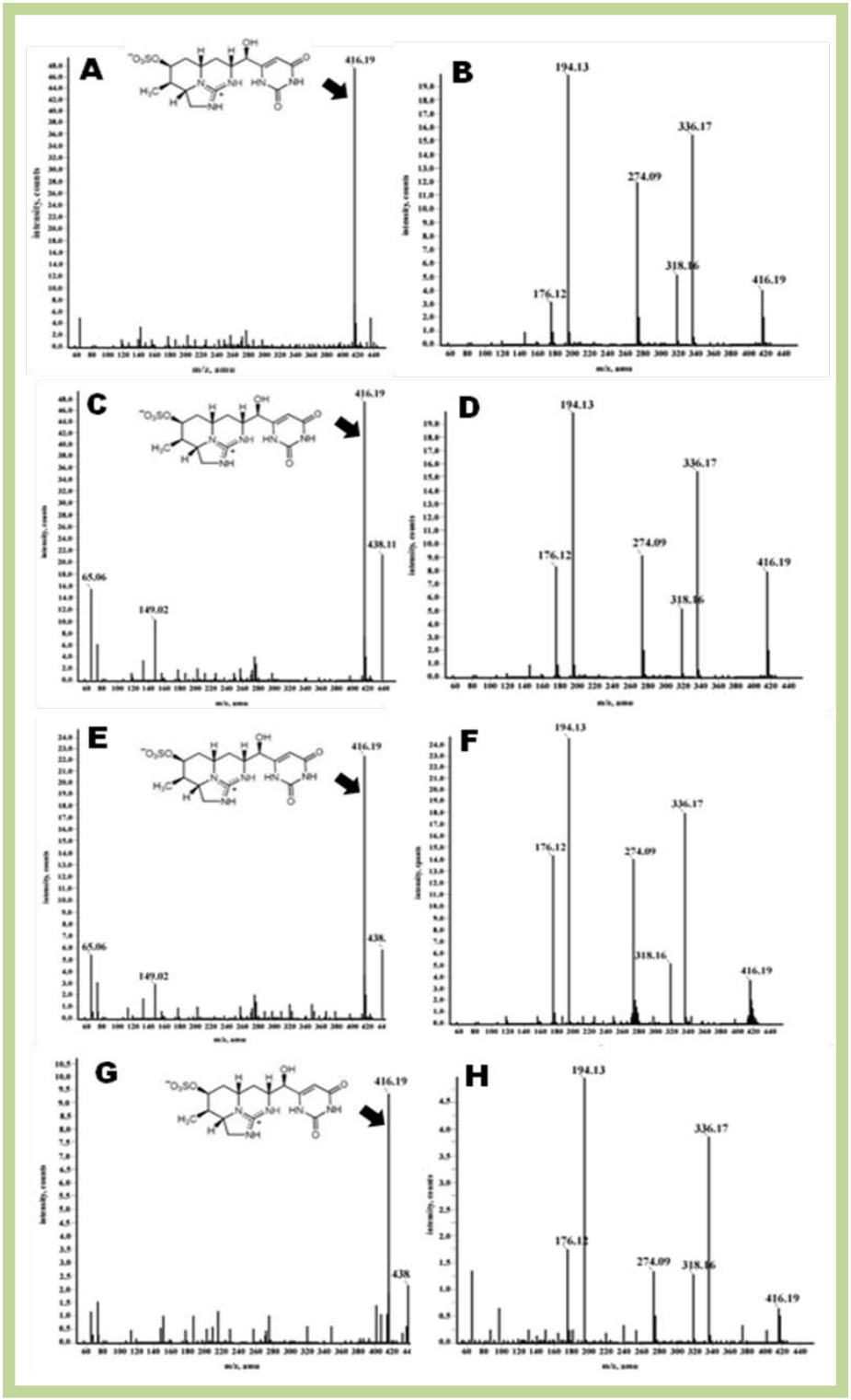
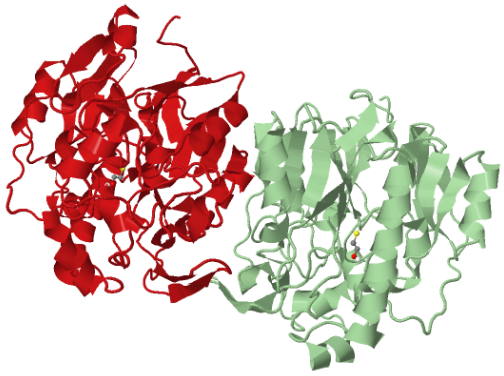


Fig.3.1.11. Full scan (A, C, E, G) and MS/MS spectrum (B, D, F, H) of the product ions of cylindrospermopsin (M+H ion, 416 *m*/*z*) in the positive ionization mode. A and B ,CYN standard; C and D, *A. ovalisporum* UAM-MAO; E and F, *A. ovalisporum* VAC^{+}; G and H, *C. raciborskii* VCC^{+}.

3.2. CHAPTER II

Characterization of *Aphanizomenon ovalisporum* amidinotransferase involved in cylindrospermopsin synthesis



Characterization of *Aphanizomenon ovalisporum* amidinotransferase involved in cylindrospermopsin synthesis

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3.2.1. Abstract

An increasing abundance of *Aphanizomenon ovalisporum* in waterbodies from diverse world regions has been reported in the last few years, with the majority of the isolated strains producing the toxin cylindrospermopsin (CYN), leading to a rise in ecological and health risks. The understanding of CYN synthesis is crucial in the control of CYN production. An amidinotransferase (AMDT) seems to be the first enzyme involved in the synthesis of CYN. In the present work, we have cloned and overexpressed the *aoaA* gene from the constitutive CYN-producer *A.ovalisporum* UAM-MAO. The recombinant purified AoaA was characterized, confirming that it is an L-arginine: glycine amidinotransferase. It shows an optimal activity between 32 to 37 ºC, at pH from 8 to 9. The activity exhibits a mixed (ping-pong /sequential) kinetic mechanism, and is inhibited by the reaction product GAA in a non-competitive manner. Mg^{2+} stimulates AoaA activity while Co^{2+} and Mn^{2+} inhibit it. AoaA conserves the critical residues of the catalytic site and substrate specificity of AMDTs, as the previously reported AMDT from *Cylindrospermopsis raciborskii* Cyr. Both proteins can be included in a new group of prokaryotic AMDTs involved in CYN production.

Keywords: cyanobacteria, cylindrospermopsin, toxin, guanidinoacetate, amidinotransferase, enzyme activity.

3.2.2. Introduction

Aphanizomenon ovalisporum is a filamentous cyanobacterium that in the last decade has become a cause for concern in fresh-water habitats, due to its ability to produce the potent alkaloid toxin cylindrospermopsin (CYN).

Several cyanobacteria species have been reported to synthesize CYN (CYN^+): *Umezakia natans* (Harada et al., 1994), *A. ovalisporum* (Banker et al., 1997, Shaw et al., 1999), *A. flos-aquae* (Preussel et al., 2006), *Raphidiopsis curvata* (Li et al., 2001) and *R. mediterranea* (McGregor et al., 2011), *Anabaena bergii* (Schembri et al., 2001), *A. lapponica* (Spoof et al., 2006), *Cylindrospermopsis raciborskii* (Hawkins et al., 1985), *Lyngbya wollei* (Seifert et al., 2007), and *Oscillatoria* sp. (Mazmouz et al., 2010). Among these CYN^+ species, the most abundant and best documented are *C. raciborskii* and *A. ovalisporum*.

CYN^+ *C. raciborskii* and *A. ovalisporum* species appear to be differentially distributed world-wide. Although, *C. raciborskii* , initially described in tropical zones, is spreading to temperate regions, its CYN^+ strains have been only found in Australia, Asia and South America (Sinha et al., 2012). In contrast, CYN^+ *A. ovalisporum* has been reported in Europe, Middle East, Australia and North America (Yilmaz et al., 2008; Kinnear, 2010,). These distribution differences could be due to distinct ecophysiological strategies for the survival of the two species (Everson et al., 2011). Mehnert et al. (2010) and Sukenik et al. (2012) warned about the invasive behavior of *A. ovalisporum* associated to global climate change. Interestingly, except in one case (Ballot et al., 2011) all strains of *A. ovalisporum* isolated so far are CYN^+. Moreover, CYN was detected under all culture conditions assayed, including different nutrient settings (Bacsi et al., 2006), temperature and light intensity (Cires et al., 2011). The presence of two transcriptional start points for *aoaA-C* genes was also reported, suggesting the existence of one constitutive promoter (Shalev-Malul et al., 2008). The concentration of CYN detected in blooms dominated by *A. ovalisporum* has usually been higher than that of CYN^+ *C. raciborskii* blooms, ranging between 9.4-18 µg CYN L^{-1} (Quesada et al., 2006, Messineo et al., 2010); but larger concentrations, up to 120 µg CYN L^{-1} in Autralia (Shaw et al., 1999) were registered.

A putative CYN biosynthetic pathway has been proposed according to isotope-labelled precursor feeding experiments and genetic data. A partial gene cluster involved in CYN

production was first characterized in *A. ovalisporum* (*aoa* genes) (Shalev-Alon et al., 2002, Kellmann et al., 2006), and later the complete gene cluster in *C. raciborskii* (*cyr* genes) (Mihali et al., 2008), *Aphanizomenon* sp. 10E6 (Stuken and Jakobsen, 2010), *Oscillatoria* sp. PCC6506 (Mazmouz et al., 2010) and *R. curvata* CHAB1150 (Jiang et al., 2012) were described. Several molecular determinants based on *aoa* (Barón-Sola et al., 2012) and *cyr* (Fergusson and Saint, 2003, Rasmussen et al., 2008) gene sequences, encoding for PKS, NRPS, AMDT or sulfotransferase (*CyrJ*) have been successfully used to discriminate between CYN^+ and CYN^- cyanobacterial strains.

Both *aoa* and *cyr* genes show high similarity, and hypothetically encode, among other proteins, an amidinotransferase (AMDT), a non-ribosomal peptide synthetase (NRPS) and a polyketide synthase (PKS). The AMDT was proposed as the first enzyme involved in CYN synthesis (Burgoyne et al., 2000; Kellmann et al., 2006; Mihali et al., 2008). AMDTs catalyze the reversible transfer reaction of an amidino group (donor) to an amine group (acceptor). The characterized AMDTs utilize L-arginine as the main amidino donor substrate, and glycine (Humm et al., 1997a, Lee et al., 2002, Muenchhoff et al., 2010, Muenchhoff et al., 2012), inosamine phosphate (Fritsche et al., 1998) or lysine (Hernandez-Guzman and Alvarez-Morales, 2001) as acceptor molecules. In general, AMDTs can use a wide variety of substrates. An exception is the AMDT from *C. raciborskii* AWT205, CyrA, the only cyanobacterial AMDT characterized to date (Muenchhoff et al., 2010, Muenchhoff et al., 2012). In effect, CyrA, encoded by the *cyrA* gene, can use only L-arginine as a donor of the amidino group, and glycine as acceptor; therefore, it is considered to be an L-arginine: glycine amidinotransferase (Muenchhoff et al., 2010).

The important contribution of *A.ovalisporun* to CYN production due to its ability to constitutively synthesize the toxin at high level, its world-wide distribution and invasive trend, and the scarce information about CYN synthesis and its regulation, lead us to study the protein encoded by the *aoaA* gene, a putative AMDT. The *aoaA* was cloned, the protein product (AoaA) overexpressed, purified and characterized biochemically, confirming its AMDT activity. Initial studies on the control of CYN synthesis by AoaA and the modulation of the AMDT activity by temperature, pH and some cations were performed. In addition, AoaA was phylogenetically compared with other AMDTs of diverse origin.

3.2.3. Materials and methods

3.2.3.1. Strains and vectors

The strain *A. ovalisporum* UAM-MAO was isolated from a Spanish pond of the Parque Juan Carlos I in Madrid (40º 27´ N; 3º 36´ W), used for various recreation activities. The mean temperature and pH in the water at the time of sampling was 22 ºC and 7.2, respectively. The strain was identified according to (Komarek and Anagnostidis, 1989), and cultured in BG_{11} medium under continuous white light (60 µmol m^{-2} s^{-1}) at 28 ºC.

Two *Escherichia coli* strains were utilized: DH5α, for cloning and sequencing the *aoa*A gene into pET28b+ vector (Novagen, Madison, WI, USA), and BL21 (DE3) used for protein overexpression.

3.2.3.2. Cloning of aoaA and overexpression of AoaA

Cyanobacteria DNA extraction and purification were carried out following the method developed by Smoker and Barnum (1988), and modified by (Neilan, 1995). The *aoa*A gene was amplified by PCR with the specific primers *aoa*A-F (5' AAAAGAATTCGATGCAAACAGGAATTGTAAATAGCTG 3') and *aoa*A-R (5' AAAAAAGCTTCAAACCTACTAAATAATGATGAAGCG 3'), containing EcoRI and HindIII restriction sites, respectively. The *aoa*A sequence described by Shalev-Alon et al. (2002) was used as template to design the primers. The PCR product and pET28b+ vector were double digested using EcoRI and HindIII enzymes (Takara) and purified from agarose gel using GFX PCR DNA Gel Band Purification Kit (GE Healthcare) for subsequent ligation. The constructed vector was firstly transformed into *E. coli* DH5α for sequence analysis and also introduced into *E. coli* BL21 (DE3) for expression as an N-terminal His_6-tagged fusion protein. Plasmid purification was performed with Zyppy™ Plasmid Miniprep Kit (Zymo Research). Expression of the recombinant His_6-tagged *aoa*A was performed in LB medium supplemented with 30 µg L^{-1} of Kanamycin and 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 28 ºC and constant agitation (200 rpm).

3.2.3.3. Purification of AoaA

Cells of the recombinant suspension culture were harvested by centrifugation (6000 *g* for 15 min at 4ºC). The pellet was suspended in chilled lysis buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, 30 mM imidazole). Cell lysis was performed by sonication for 10 min in an ice bath with a Braun Labsonic 2000*,* using a 100W needle probe. The crude extract was centrifuged 12000 *g* for 15 min, and the supernatant loaded onto a HiTrap Chelating Column (GE Healthcare, Waukesha, WI, USA) for protein purification, according to manufacturer instructions. An imidazole gradient (100-500mM) was used for protein elution, and the eluted fractions were analyzed in SDS-PAGE gels followed by Coomasie blue staining. The fractions containing the purified recombinant protein AoaA were pooled and desalted with an Amicon filtration unit 10 kDa (Amicon® Ultra). The buffer (50 mM HEPES, pH 7.5, 10% glycerol, 10 mM dithiothreitol) was used for protein dilution and storage at -80ºC. Protein concentration was determined by the (Bradford, 1976) using bovine serum albumin as a protein standard.

3.2.3.4. Protein sequence analysis

The protein band corresponding to 50.2 kDa on SDS-PAGE was excised (Fig. 1), destained and extracted. The recombinant protein AoaA was digested with trypsin, and the resulting peptides analyzed by MALDITOF MS. The AoaA amino acid sequence obtained was submitted to BLAST (GenBank accession number AEQ64884) for sequence comparison.Clone Manager Software was used to calculate the predicted molecular mass and theoretical isoelectric point (Ip). Sequence alignments were performed by MEGA5 software (Tamura et al., 2011).

3.2.3.5. Phylogenetic Analysis

Twenty nine AMDT amino acid sequences from the GenBank database were aligned, using the ClustalW tool from MEGA5 software (Tamura et al., 2011). A Neighbor-joining phylogenetic tree (1000 bootstrap) was constructed, following the substitution model of Jones-Taylor-Thornton (Jones et al., 1992).

3.2.3.6. Assay of amidinotransferase activity

The AMDT activity was determined colorimetrically by measuring the production of ornithine with ninhydrin reaction at low pH (Van Pilsum, JF. et al., 1970), following the modifications of (Muenchhoff et al., 2010). Briefly, unless specified, the reaction mixture contained in a final volume of 300 µL: 50 mM Tris-HCl pH 8.5, 20 mM L-arginine and 20 mM glycine as substrates, and 8-12 µg of purified AoaA. The reactions were performed in triplicate at 30 ºC during 30 min. The specific AMDT activity is expressed as µmole ornithine formed per min and per mg protein.

3.2.3.7. Identification of the products of the AMDT activity

The AMDT reaction of AoaA was carried out in a final volume of 4 mL, containing 20 mM L-arginine and/or glycine. After incubating for 3 h at 30 ºC, AoaA was removed using a 10 kDa Amicon filtration unit (Amicon® Ultra). The filtrate and standard compounds (Sigma-Aldrich™) were analyzed by LC ESI-MS. The chromatographic separation was carried out by HPLC (model 1100 Agilent Technologies), using the Zorbax C18 XDB 5 µm (50 mm x 2.1 mm) column and a mobile phase gradient. The mobile phase consisted of two eluents: A, water with 0.1% acetic acid; and B, acetonitrile with 0.1% acetic acid. The continuous gradient performed during 15 min was: t_0, A=95%; t_{15}, A=5%. The flow rate was 0.5 mL/min, and the injection volume 5 µL. The ESI-MS analyzer was a Q-TOF QSTAR AB SCIEX operated in the positive ionization mode, under the following conditions: ion spray voltage: 5.5 kV; ion source gas pressure, 1 and 2.5 psi; two declustering potentials , 3 and 15 V; and focusing potential, 210 V.

3.2.3.8. Kinetic analysis of the AMDT reaction

The kinetic parameters of the AMDT reaction were obtained by nonlinear regression analysis with the enzyme kinetics module of GraphPad prism 5.03. All kinetic analyses were repeated at least three times with reproducible results.

3.2.3.9. Assay of AMDT inhibition by guanidinoacetate

AoaA activity was measured by adding guanidine acetate (GAA) at different concentration (1- 30 mM) to the AMDT reaction mixtures that included varied concentration of glycine (0, 1, 3, 6, 15, 20 mM). 30 mM Arginine was used in all cases. The reactions were performed in triplicate. Other conditions were as stated above for AMDT assay (3.2.3.6).

3.2.3.10. Assay of AMDT activity and stability of AoaA at different pH and temperature

The AMDT activity of purified AoaA was determined at 30 °C in 100 mM buffers of pH 5.5 to 10 (pH 5.5–6.5, Mes-NaOH; pH 7–7.4, K_2HPO_4-KH_2PO_4; pH 8–9, Tris-HCl; and pH 9.5–10, Ches-NaOH). The pH stability of AoaA was evaluated by pre-incubating AoaA at 30 ºC with the different buffers during 1h, and measuring the AMDT activity every 15 min.

The effect of temperature on AMDT activity of AoaA was determined by measuring the amount of ornithine formed at pH 8.5 at various temperatures, ranging from 15 to 50 ºC. The temperature stability was analyzed by pre-incubating AoaA at pH 8.5 and at 15- 45 ºC during 1h, and measuring the AMDT activity every 10 min.

3.2.3.11. Assay of cation influence on AMDT activity of AoaA

To assess the effect of metal ions on AoaA activity, various divalent cations (Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+}) were added to the AMDT reaction mixture at different concentrations (0.01, 0.1, 1 and 5 mM). At the end of the reaction, ethylenediaminetetraacetic acid (EDTA) was added to a final concentration of 0.1mM, and the reaction mixture centrifuged (12000 *g* for 10 min). The supernatant was used to measure the ornithine formed during the reaction. All assays were performed in triplicate.

3.2.4. Results

3.2.4.1. Gene expression and purification of the recombinant protein AoaA of A. ovalisporum

The recombinant AoaA protein was purified, reaching more than 95 % purity, as shown by SDS-PAGE (Fig. 3.2.1). The mean yield of the purified AoaA was 11.2 mg per liter of culture. The molecular mass predicted from the gene sequence was 45.7 kDa, and the calculated Ip was 5.79. In the SDS-PAGE gel (Fig. 3.2.1) the molecular mass appeared slightly larger (50.2 kDa), due to the additional 6 His residues and the extra encoded amino acids from the vector.

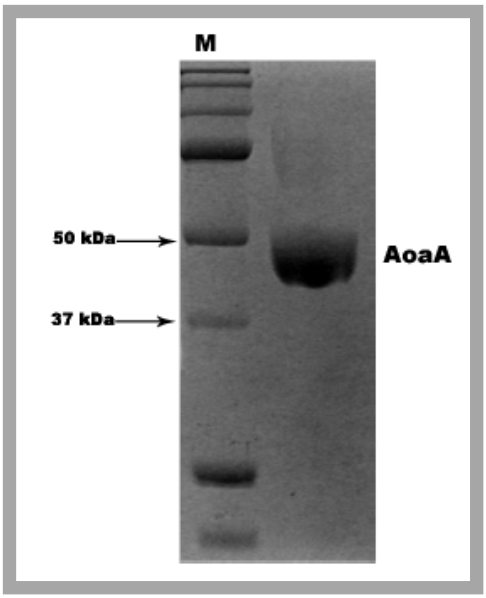

Fig.3.2.1. SDS-polyacrylamide gel electrophoresis of purified AoaA from *A. ovalisporum* UAM-MAO. M.Molecular weight marker.The Recombinant AoaA was expressed in *E. coli* BL21 (DE3), and contained an N-terminal 6-His tag and extra vector encoded amino acids.

The recombinant purified AoaA was sequenced by MS fingerprinting after trypsin digestion. More than 90% of the sequence was obtained, confirming a non-truncated form of the expected protein, a putative AMDT. Alignment of AoaA, CyrA, human AGAT, Amt1 (from *Pseudomonas syringae*) and StrB1 (from *Streptomyces griseus*) sequences showed good homology among these proteins, especially between AoaA and CyrA, with approximately 96 % identity (Fig. 3.2.2). The three amino acids related to the catalytic site of human AGAT (Humm et al., 1997b) , Asp^{254}, His^{303} and Cys^{407}, are conserved in AoaA (Asp^{197}, His^{248} and Cys^{356}). As in CyrA (Muenchhoff et al., 2010),

two residues of the human AGAT catalytic site, Asn[300] and Met[302], are substituted by Phe[245] and Ser[247] respectively. Nevertheless, several amino acids are not conserved in the AoaA sequence.

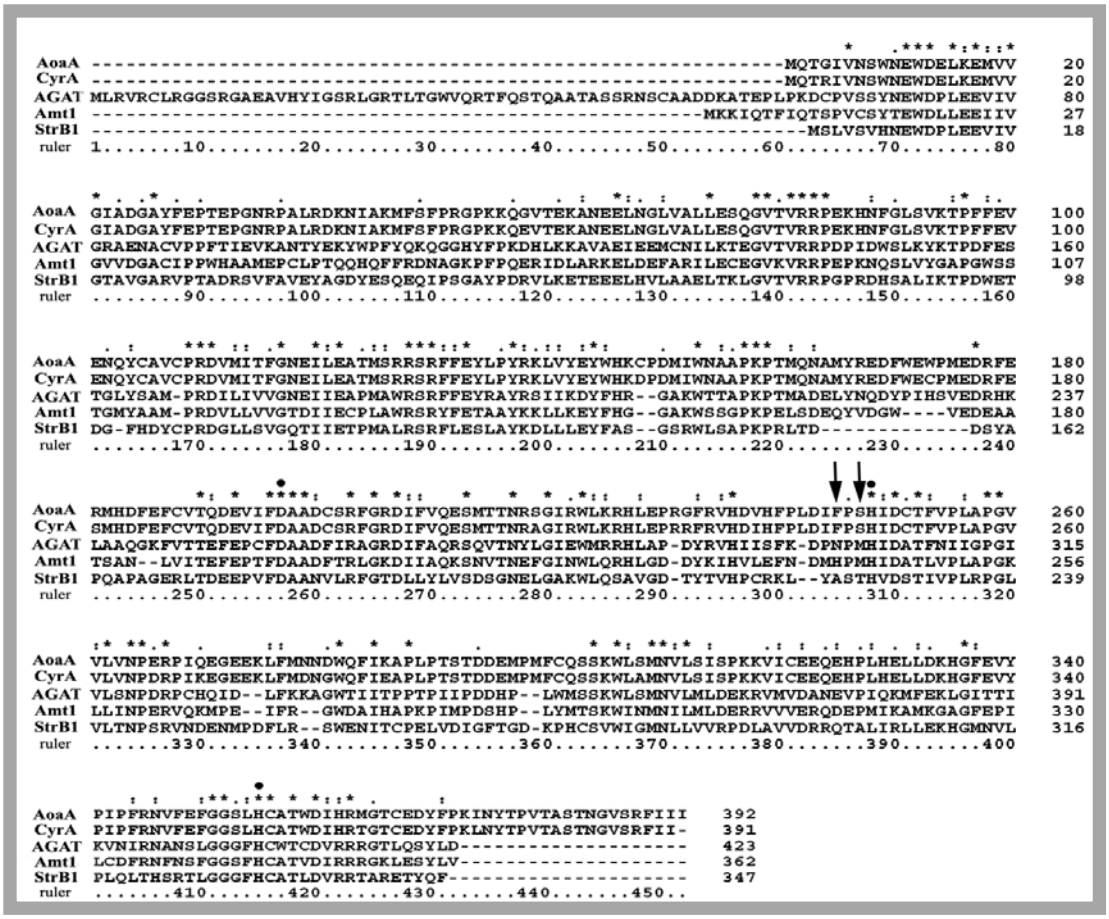

Fig.3.2.2. Alignment of AMDT amino acid sequences obtained from databases. Fully conserved amino acids (*). Residues with both conserved size and hydropathy(•). Residues either conserved size or hydropathy(:). Conserved amino acid within the catalytic site (arrows). Only AoaA and CyrA conserved amino acid in the active site. (*). AoaA: *A. ovalisporum* UAM-MAO. CyrA: *C. raciborskii* AWT205. AGAT: *Homo sapiens*. Amt1: *Pseudomonas syringae* pv. *phaseolicola*. StrB1: *Streptomyces griseus*.

3.2.4.2. Phylogenetic analysis

To explore the phylogenetic relationship of AoaA with other AMDTs, its amino acid sequence was compared with those of 28 AMDTs from different origins found in Genbank databases (Fig. 3.2.3). Most of the proteins referred to putative AMDTs; only in 5 instances the AMDT activity has been proven. The resulting phylogenetic tree (Fig. 3.2.3) comprises six clusters. Cluster I contains only AMDTs from CYN^+ species, AoaA, and CyrA being integrated in this group. Cluster II includes AMDT sequences

from *Streptomyces* species (StrB1) together with one protein from the cyanobacterium *Oscillatoria* sp PCC 6506, which presents two types of AMDTs. Cluster III groups animal AMDTs, including the well-studied human AGAT. Cluster IV collects protein sequences from saxitoxin-producing cyanobacteria from diverse origins, and two hypothetical AMDTs. Clusters V and VI are quite separated from the previous clusters. Cluster V comprises putative AMDTs from green algae species. Cluster VI, rather distant from the others, incorporates putative AMDTs from Archae domain.

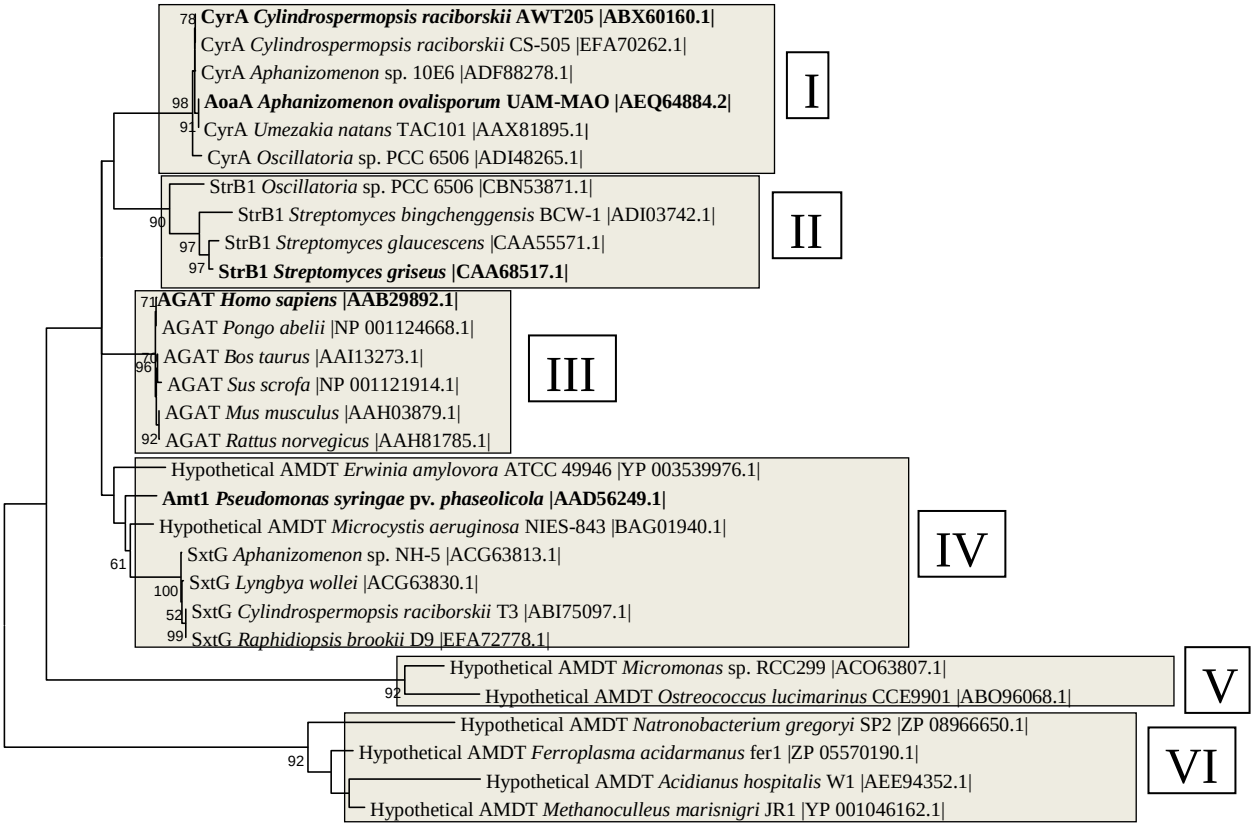

Fig.32.3. Dendrogram of AMDT amino acid sequences. Twenty eight sequences from the GenBank database, besides that of the AoaA product were used. The neighbor-joining method was applied with a bootstrap value of 1000, following the substitution model of Jones-Taylor-Thornton. Clusters are indicated by roman numbers. In bold, sequences from already characterized proteins.

The phylogenetic tree shows the close relationship not only between the recombinant purified AMDTs from *C. raciborskii* AWT205 (CyrA) and *A. ovalisporum* UAM-MAO (AoaA), but also among putative AMDTs from other CYN^+ cyanobacteria: *C.*

raciborskii CS-505, *Aphanizomenon* sp. 10E6, *Oscillatoria* sp. PCC6506 and *Umezakia natans* TAC101. However, another AMDT from *Oscillatoria* sp. PCC6506 is also close to that of *Streptomyces* (cluster II), and clearly appears separated from the CYN^+ cyanobacteria group (cluster I).

3.2.4.3. Analysis of AMDT activity

Considering the great similarity of the amino acid sequence in AoaA and CyrA, and knowing that CyrA was an AMDT, it seemed obvious to confirm that AoaA was also an AMDT. The recombinant purified AoaA showed AMDT activity, as revealed by the formation of ornithine and GAA when it was incubated with L-arginine and glycine (Fig. 3.2.4A). However, GAA was not detected when glycine was omitted in the reaction mixture (Fig. 3.2.4B), strongly indicating that both glycine and L-arginine were acting as AMDT substrates, and suggesting a ping-pong mechanism in the enzymatic activity.

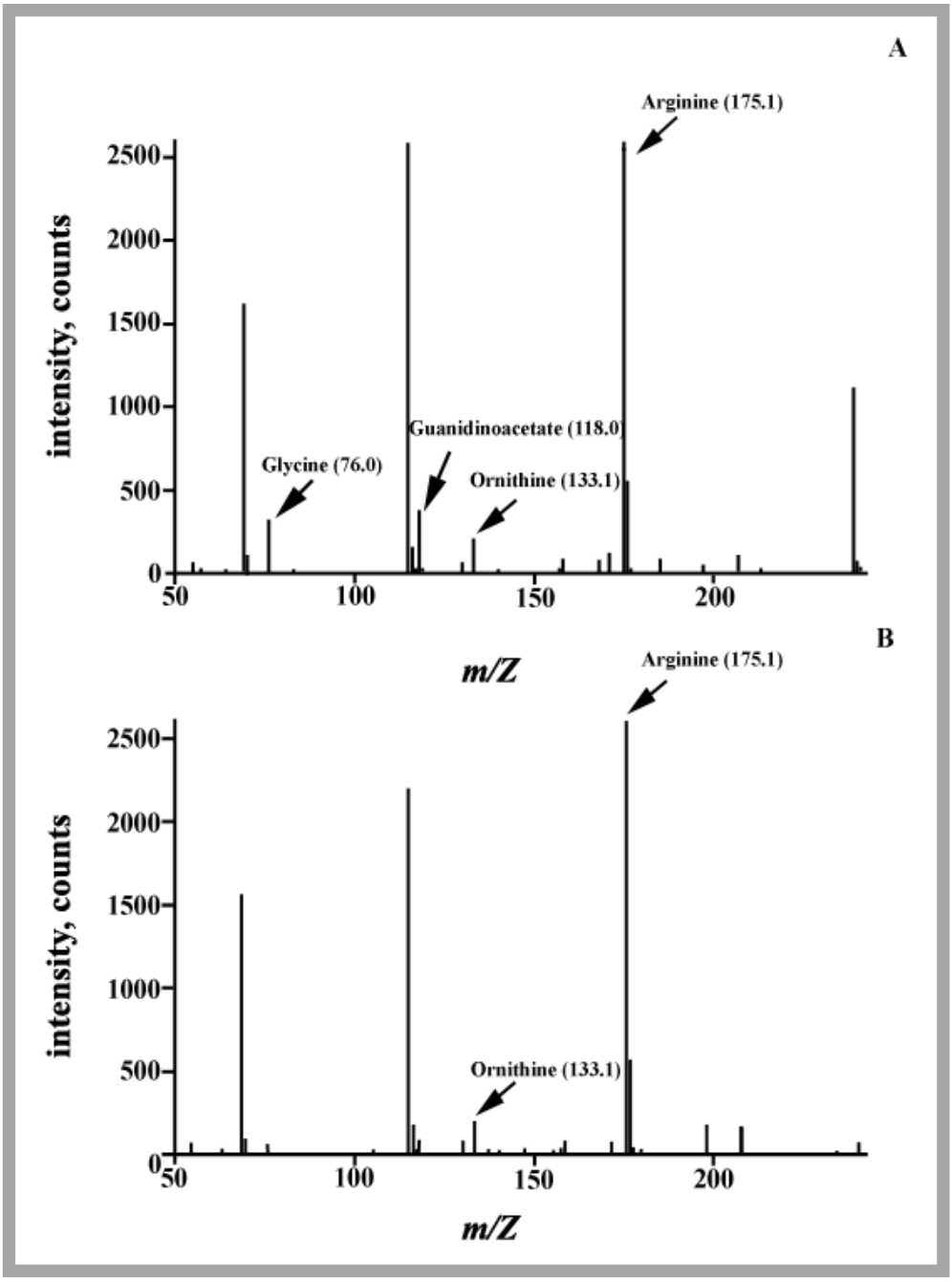

Fig.3.2.4. ESI-MS spectra of substrates and products in the AMDT reaction of AoaA. **A**: L-arginine (175.1) and glycine (76.0) were used as substrates. Ornithine (133.1) and guanidinoacetate (118.0) were the reaction products. **B**: L-arginine (175.1) was used as unique substrate. Ornithine (133.1) was the reaction product.

At constant concentrations of L-arginine and glycine (20 mM each), the AMDT activity of AoaA was linear over a time period of 45 min. Linearity was also observed with respect to enzyme quantity between 2.5 and 20 µg. The kinetic parameters of AoaA AMDT found at 30 ºC and pH 8.5 were: V_{max}, 0.62; Km_{Arg} and Km_{Gly} 0.74±0.2 and 5.68±0.7, respectively (Table 3.2.1).

Table 3.2.1. Kinetic parameters of different amidinotransferases

	AoaA[(1)]	CyrA[(2)]	AGAT[(3)]	Gm AMDT[(4)]
V_{max} (µmol·min^{-1}·mg^{-1})	0.62	1.05	0.44	nd
K_m L-arginine (mM)	0.74 ± 0.2	3.5 ± 1.14	2 ± 0.5	3.8
K_m glycine (mM)	5.8 ± 0.7	6.9 ± 2.7	3 ± 1	0.89
Optimal temperature (°C)	32-37	32	37	37
Optimal pH	8.5-9	8.5	7.5	9.5

Gm, *Glycine max;* References: (1) This study; (2) (Muenchhoff et al., 2010); (3) (Fritsche et al., 1997); (4) (Lee et al., 2002)

In the kinetics representation of Fig.3.2.5, the lines intersect at the left of the y-axis and bellow the x-axis indicating that binding of one substrate to the enzyme diminishes the affinity for the other substrate; therefore, AoaA seems to bind to glycine and L-arginine, in a random way before releasing the first reaction product, suggesting a random sequential mechanism. However, ornithine was also produced when only L-arginine was present (Fig. 3.2.4 B). This result would not support a typical random sequential mechanism. Thus, taken together the data of Figs. 3.2.4 and 3.2.5 suggest a hybrid random sequential-ping/pong mechanism for the AMDT activity of AoaA.

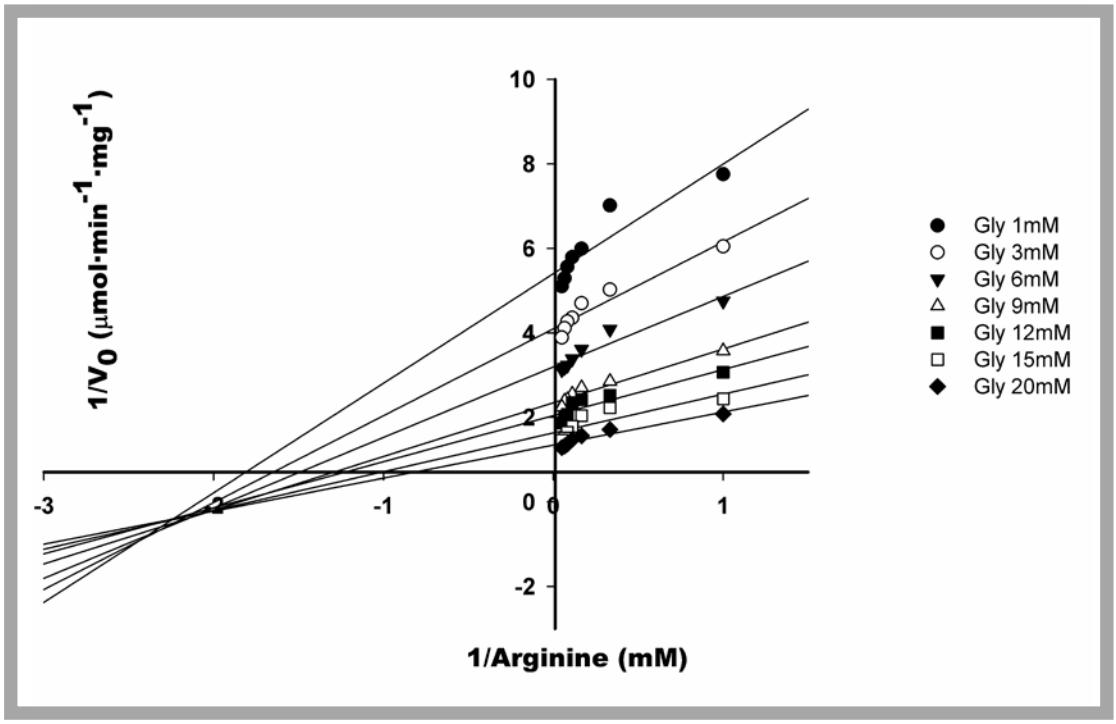

Fig.3.2.5. AMDT kinetics of the AMDT reaction of AoaA**.** Double reciprocal plot of the initial enzyme velocity versus the L-arginine concentration at the glycine concentrations indicated in the graph insert.

When GAA was included at different concentrations in the AMDT reaction mixture using various glycine concentrations, the double reciprocal plot representation the group of the resulting lines intersect on the x-axis (Fig. 3.2.6), indicating a non-competitive inhibition effect by GAA of the AoaA AMDT activity.

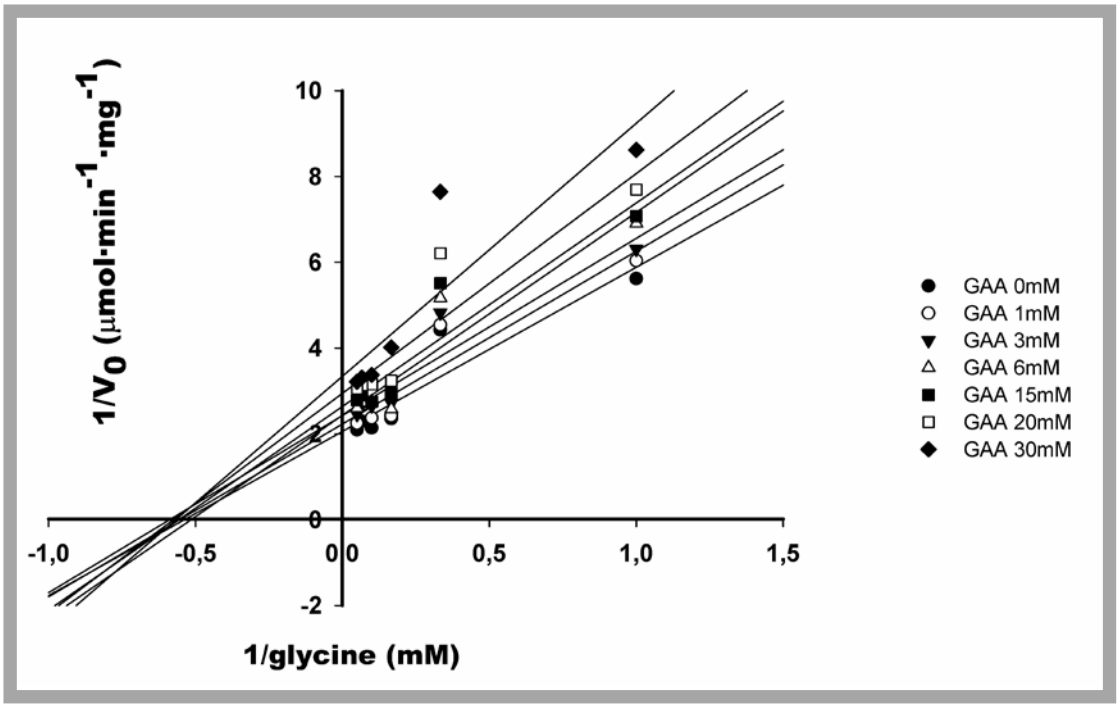

Fig.3.2.6. Effect of guanidinoacetate on AMDT reaction of AoaA. Double reciprocal plot of the initial velocity versus L-glycine concentration at the GAA concentrations indicated in the graph insert. L-arginine was maintained at saturated concentration (30 mM).

3.2.4.4. Influence of diverse factors on AoaA activity and stability

The formation of cyanobacterial blooms depends on physical and chemical environmental changes; therefore, CYN production would also probably be influenced by those changes. On this basis we thought it would be interesting to evaluate the AMDT activity and stability of AoaA at different pH, temperature and cation concentration.

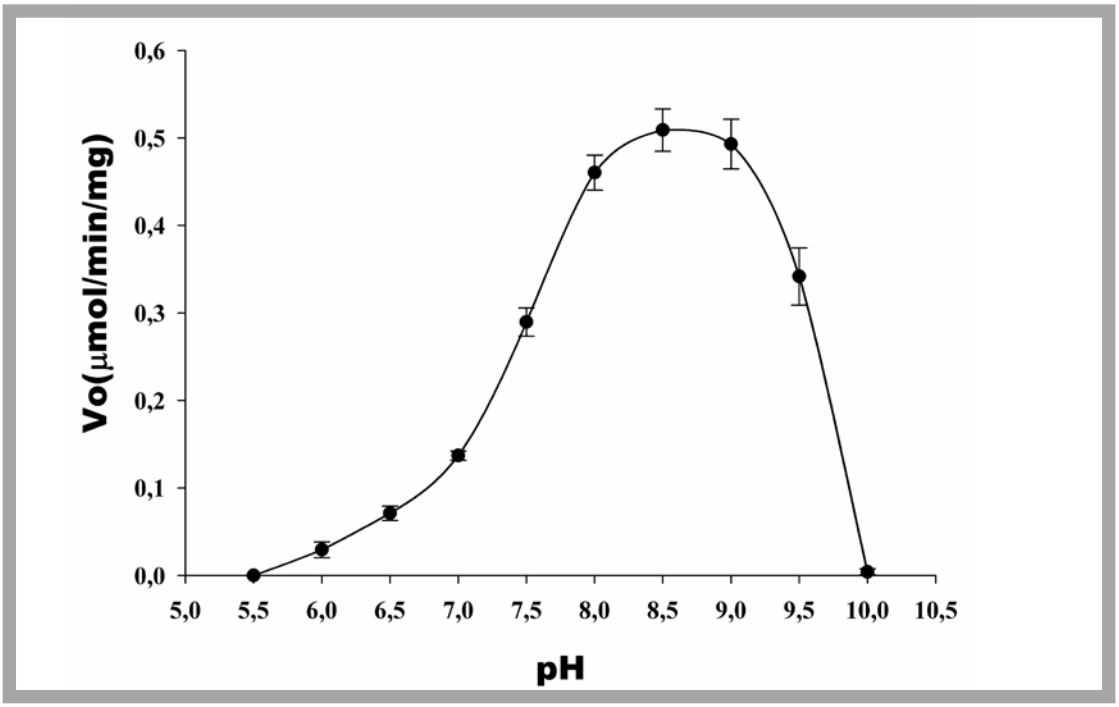

Fig.3.2.7. Effect of pH on AMDT activity of AoaA. The assay was performed at 30 ºC during 30 min in the pH range 5.5-10. Data are given as means (n=3), and error bars represent SD.

The AMDT activity and stability of the recombinant purified AoaA were assayed in the pH range 5.5-10, and it was found that both were not significantly affected by usual pH values in water ecosystems. Although the highest activity was attained between 8.5- 9 (Table 3.2.1), almost 60% of the activity remained at pH 7.5 and 9.5 (Fig. 3.2.7). The maximum stability, tested by AMDT activity after preincubation of AoaA for 1 h at 30 ºC, was found at pH 7.5 (Fig. 3.2.8A); but more than 50% of the initial activity was retained at 6.5 and 9.5 (Fig. 3.2.8A and 3.2.8 B).

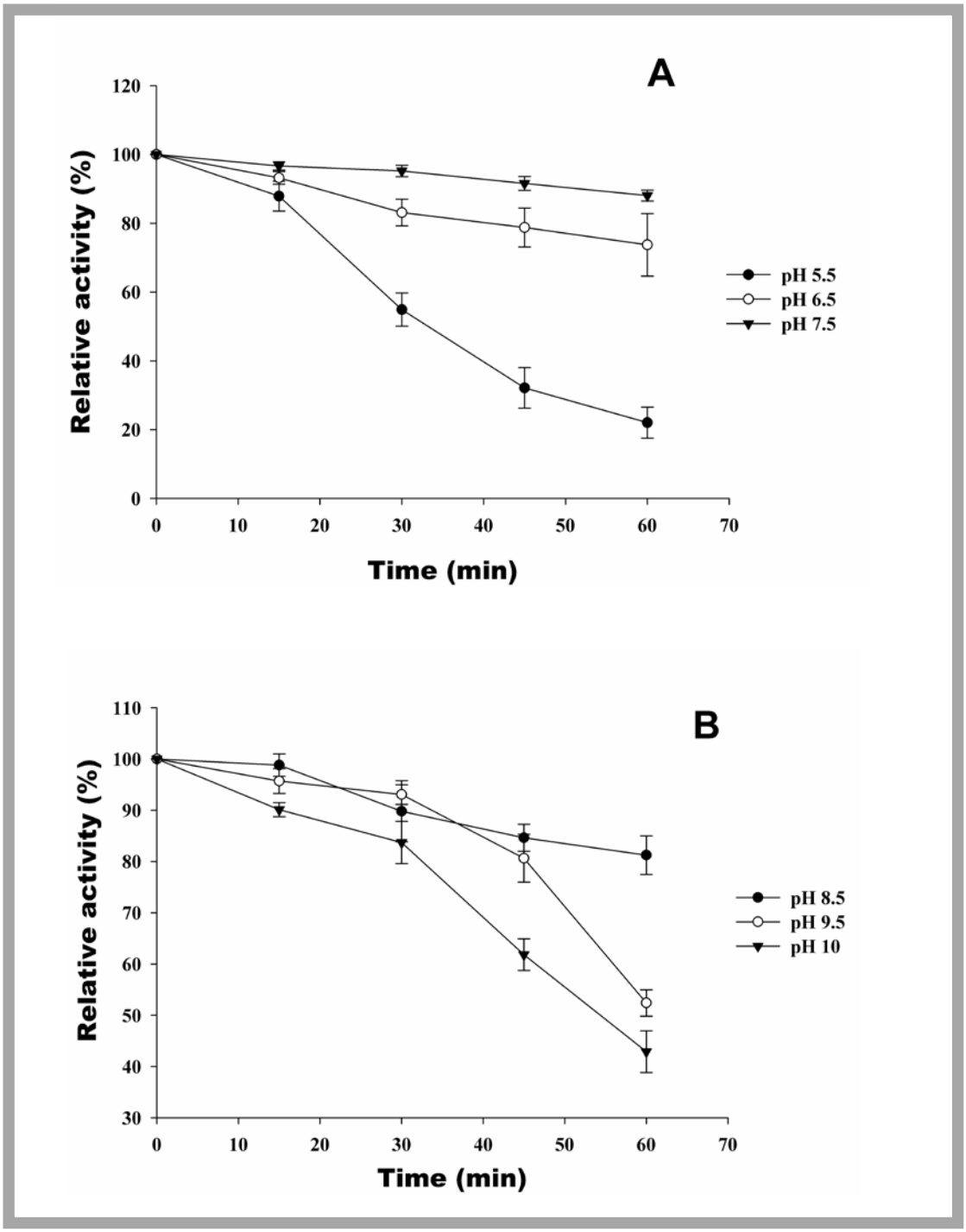

Fig.3.2.8. Characterization of AoaA stability under different pH. (A), pH5-7.5; (B), pH 8.5-10. Purified AoaA was preincubated at 30 ºC and several pH buffers for varying time periods (0-60 min) and then assayed for residual activity. Data are given as means (n=3), and error bars represent SD.

Thermal activity was tested between 15 and 50 ºC. Special care was taken to perform the assays on temperature and pH activity within the time range in which the activity

was linear, to prevent side-effects of AoaA stability. Fig. 3.2.9 shows that although the optimal temperature was at 32 ºC, subtle differences were found between 30 ºC to 37 ºC (Table 3.2.1) with more than 70 % activity remaining between 25-30 ºC.

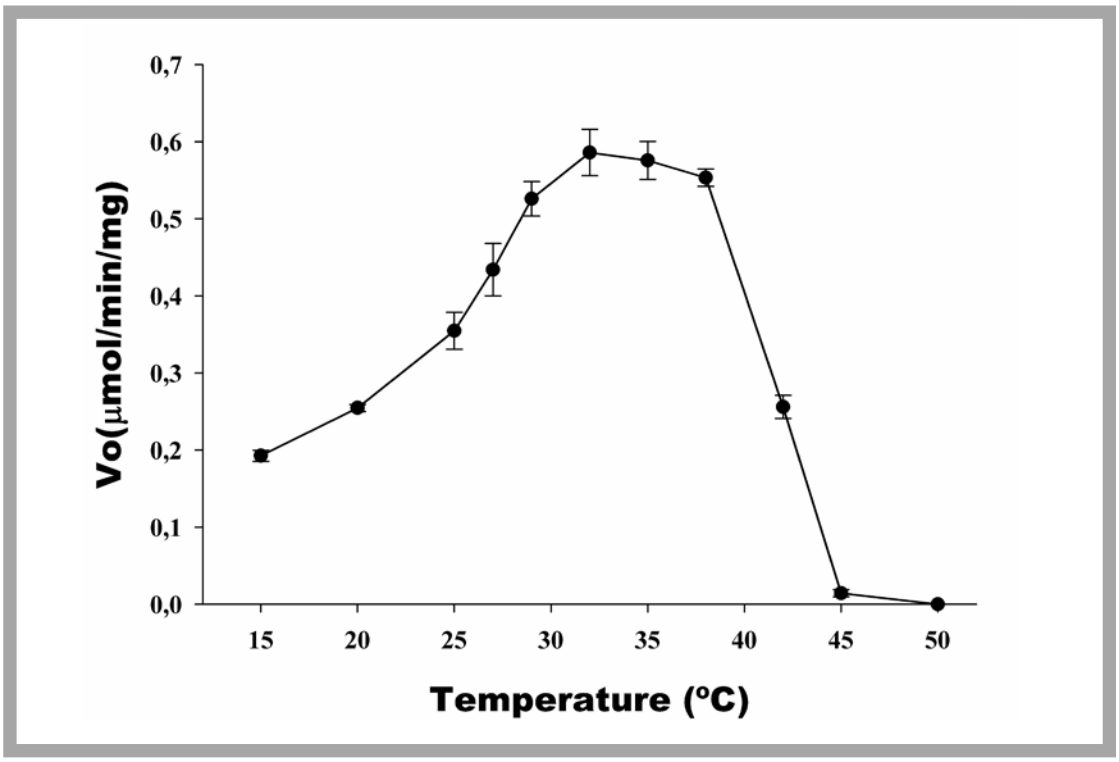

Fig.3.2.9. Effect of temperature on AMDT activity of AoaA. The assay was performed with Tris-HCl pH 8.5, during 30 min at the temperature range 15-45 ºC. Data are given as means (n=3), and error bars represent SD.

In relation to thermal inactivation, the maximum stability was found between 25-30 ºC, since at both temperatures more than 90% activity remained after 60 minutes (Fig. 3.2.10 A). At 40 ºC, 75% of activity was lost after 30 min, and at 45 ºC AoaA was completely inactivated, after 10 min (Fig. 3.2.10 B).

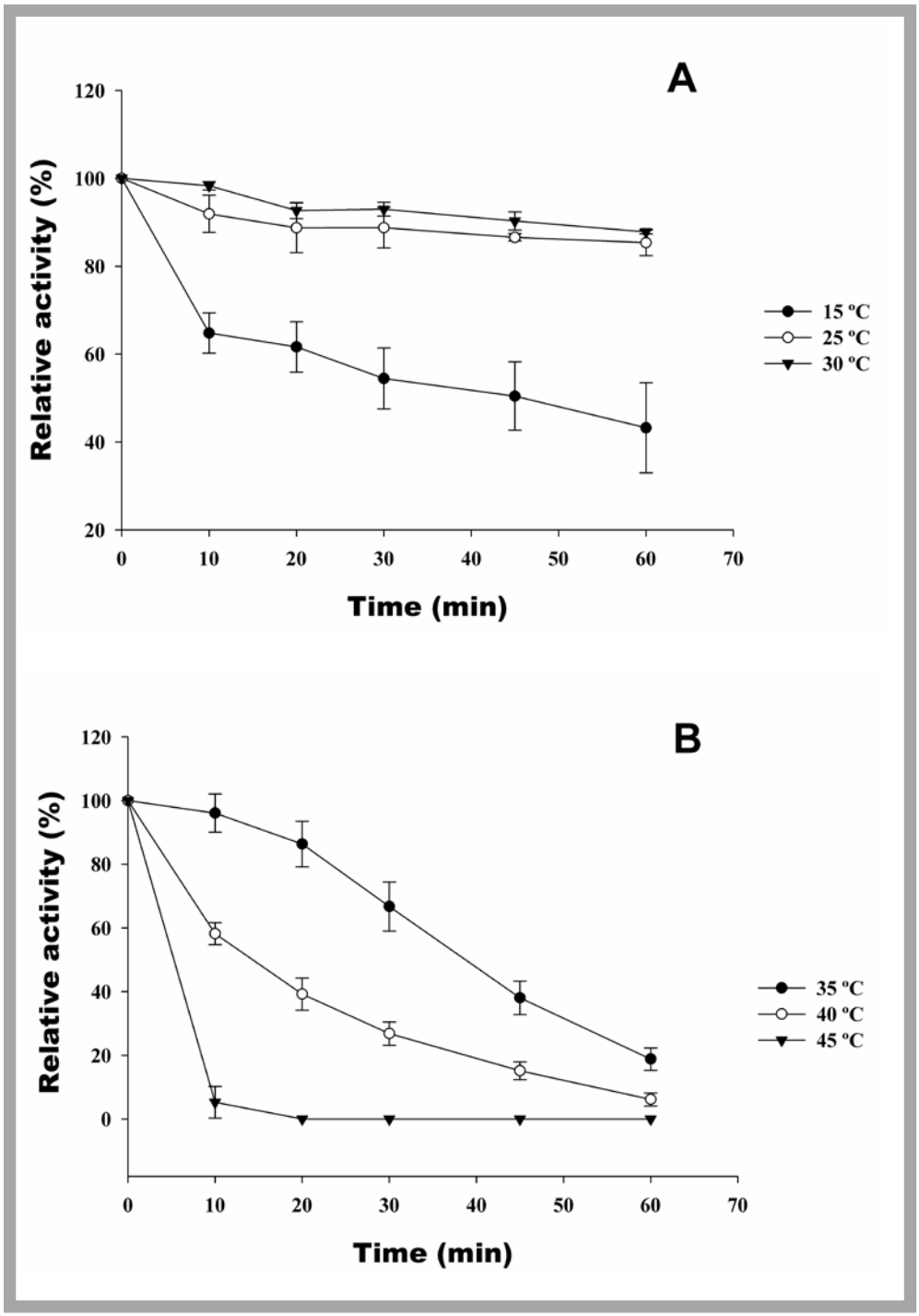

Fig.3.2.10. Characterization of AoaA stability under different temperatures: (A) 15-30 °C; (B) 35-45°C. Purified AoaA was preincubated in Tris-HCl pH 8.5, at different temperatures for varying time periods (0-60 min), and then assayed for residual activity. Data are given as means (n=3), and error bars represent SD.

Fluctuations of ion concentration in dynamic water systems are usual events. On the other hand, divalent cations intervene in numerous enzymatic activities, being able to modulate them in a direct or indirect way. For those reasons, the AMDT activity of AoaA was determined in the presence of various divalent cations at different

concentrations (0.01, 0.1, 1 and 5 mM). The cations tested were Ca^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}, and Ni^{2+}. Fe^{2+}, Ni^{2+} and Ca^{2+} did not affect significantly the AMDT activity; however, Co^{2+} at all concentrations assayed produce inhibition, and Mn^{2+} at 5 mM inhibited the activity 80 % (Fig. 3.2.11). Curiously, Mg^{2+} at 0.1 mM showed a clear stimulatory effect, enhancing the activity by around 30%.

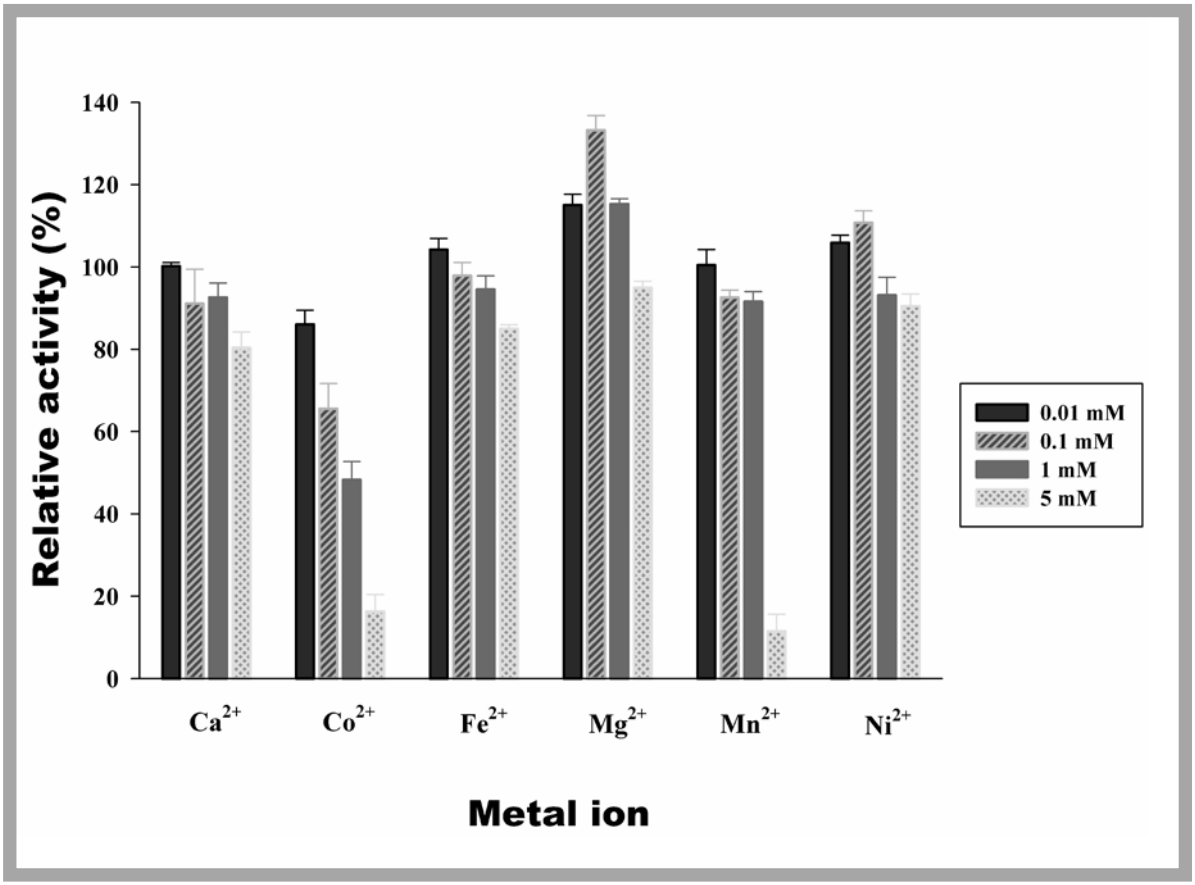

Fig.3.2.11. Effect of divalent cations on AMDT activity of AoaA. Enzyme activity was measured in the presence of different concentrations (0.01-5 mM) of the divalent cations indicated in the graph during 30 min. Data are given as means (n=3) and error bars represent SD.

3.2.5. Discussion

Cyanobacterial AMDTs are involved in the synthesis of cylindrospermopsin and saxitoxin, but until now only the AMDT from the CYN producer *C. raciborskii* AWT205, CyrA, has been characterized (Muenchhoff et al., 2010). To widen the understanding of CYN synthesis, the *aoaA* gene of the CYN^+ *A. ovalisporum* strain UAM-MAO, isolated from a Spanish artificial lake, was cloned, overexpressed, and the recombinant protein, AoaA, characterized.

AoaA appears to be very similar to CyrA. In both proteins the amino acid sequence is almost identical, and two residues of the human AGAT catalytic site, Asn^{300} and Met^{302}, are substituted by Phe^{245} and Ser^{247} respectively (Fig. 3.2.2). Recently, it has been stated (Muenchhoff et al., 2012) the role of these two residues in CyrA with the narrow substrate specificity previously observed by Muenchhoff et al. (2010).

Phylogenetically, AoaA is closely related not only to CyrA but to other putative AMDTs of different CYN^+ cyanobacteria (Fig. 3.2.3). The phylogenetic analysis shows other interesting data. For example, the position of one of the hypothetical *Oscillatoria* sp PCC6506 AMDTs appears to be closer to *Streptomyces* AMDT than to those of CYN-producing cyanobacteria. Interestingly, this *Oscillatoria* AMDT might use inosamine phosphate as amidino acceptor instead of glycine, as it is the case of AMDTs of the CYN producers. It is also worthwhile to mention the great phylogenetic proximity of AoaA to vertebrate AMDTs (Fig. 3.2.3).

AoaA, like CyrA, can use both glycine and arginine as substrates, giving rise to ornithine and GAA as reaction products (Fig. 3.2.4). The V_{max} and *Km* for glycine of the two AMDTs is similar; but the affinity for arginine is higher in AoaA (Table 3.2.1). For more than a decade glycine has been considered as a substrate in CYN synthesis, since in experiments with isotope labelled glycine it was shown that this amino acid was incorporated in the CYN molecule (Burgoyne et al., 2000). However, in the same experiments L-arginine was not incorporated in the toxin, suggesting that it might not be an adequate substrate in CYN synthesis. Our data (Fig. 3.2.4A) along with those of Muenchhoff et al. (2010) seem to contradict that suggestion, as L-arginine is clearly a substrate in the AMDT reaction catalyzed by AoaA (Fig.3.2.4A).

The likeness between AoaA and CyrA also applies to the enzymatic mechanism involved in the AMDT reaction, a mixed ping-pong-random sequential system. The hybrid system in AMDT of AoaA can be taken from the data of the double-reciprocal plot of the activity with L-arginine as the varied substrate (Fig. 3.2.5), along with the analysis of the reaction products, ornithine or GAA, found in the presence of either, L-arginine plus glycine (Fig. 3.2.4A) or solely L-arginine (Fig.3.2.4B) respectively. Therefore, the mixed kinetic system of AoaA and CyrA observed with glycine and L-arginine would differentiate these cyanobacterial AMDTs from AGAT (Fritsche et al., 1997), exhibiting a clear ping-pong mechanism.

We have also observed a non-competitive inhibition of AoaA by GAA using glycine as a varied substrate, in support of a sequential mechanism (Fig.3.2.6). GAA might bind to AoaA out of the catalytic center inducing conformational changes that would decrease AoaA activity. Therefore, GAA could also be considered as a product inhibitor and regulator of AMDT activity, as other previously described compounds produced in different metabolic pathways such as ornithine (Sipila, 1980; Muenchhoff et al., 2010) , creatine (Guthmiller et al., 1994), putrescine or spermidine (Lee et al., 2002). Ornithine was identified as a strong inhibitor of CyrA activity (Muenchhoff et al., 2010) and rat AMDT (Sipila, 1980), but the type of inhibition seems to be different from that observed with GAA in AoaA (Fig. 3.2.5). Ornithine caused a partial mixed inhibition of CyrA (Muenchhoff et al., 2010) and a competitive inhibition of vertebrate AMDT (Sipila, 1980). It would be worthwhile to assay if GAA inhibits CyrA and vertebrate AMDT activity, and if ornithine inhibits AoaA activity

The optimal temperature for maximal activity (T_{max}) and the highest stability of AoaA were between 32-37 ºC (Fig. 3.2.8), and 25-30 ºC , respectively (Fig. 3.2.9A). The pH for maximal activity and highest stability were between 8.0-9.0, and 7.5, respectively. Considering the mean temperature and pH registered in the water body from which the *A. ovalisporum* UAM-MAO under study was isolated (22 ºC and pH 7.2), it appears that AoaA was acting under suboptimal conditions; yet, the protein would be stable, a fact that could compensate the low activity in CYN production. It would be interesting to study whether there are differences between the optimal temperature and pH for activity and stability of AoaA in *A. ovalisporum* strains isolated from other ecosystems.

In general, the temperature and pH values for maximal activity and stability obtained with AoaA differed from those with CyrA, since in CyrA the activity appeared to be optimal at 32 ºC and pH 8.5, and the highest stability at pH 6.5. The origin of those differences might be attributed to the distinct amino acids outside the active sites in the two proteins (Fig. 3.2.2).

AoaA activity was affected by divalent cations, normally present in water habitats and the majority utilized by cyanobacteria for structural or catalytic purposes. Among the six cations assayed, Ca^{2+}, Co^{2+}, Fe^{2+} , Mg^{2+}, Mn^{2+} and Ni^{2+}, the most remarkable effects were observed with Mg^{2+} and Co^{2+}. Mg^{2+} enhanced the activity at the lower concentrations used (0.01-1 mM) and had no effect at the highest concentration (0.5 mM). Co^{2+} at all concentrations tested (0.01-5 mM) inhibited AoaA activity, the

inhibition being 30% at 0.1 mM, and 50% at 1 mM (Fig. 3.2.11). The highest concentration (5mM) of Mn^{2+} and Ca^{2+} was inhibitory. Few data are available on the effect of metal ions on purified AMDTs. While *Glycine max* AMDT activity was not affected by monovalent and divalent cations (Lee et al., 2002), recombinant AGAT enzyme was strongly inhibited by Hg^{2+} (1 mM), Zn^{2+} (1 mM) and Ni^{2+} (10 mM) (Humm et al., 1997b). Since in our experiments the cations were only present during the AMDT reaction assay, it seems reasonable to think that the effect observed is direct on AoaA and does not result from acting on other enzyme activities involved in cyanobacteria metabolism that could in the end affect AoaA. Several questions arise from the cation data of Fig 4.11, including: i) how Mg^{2+} and Co^{2+} can act on AoaA activity?; ii) are the intracellular concentration of these two ions enough to justify the effects observed?; and iii) could the different cations exhibit a synergistic, or antagonic effect? Our results do not allow drawing any conclusion on the three posed questions. However, it could be hypothesized that Mg^{2+} and Co^{2+} effects are related to the dimer or tetramer structure conformation that active AMDTs could adopt (Humm et al., 1997a; Muenchhoff et al., 2010). On the other hand, the intracellular Mg^{2+} concentrations that would be required to enhance the AMDT activity of AoaA appear to be normal in cyanobacteria. In effect, Mg^{2+} not bound to chlorophyll has been reported to accumulate in cyanobacterial cells at concentrations in the mM range (Utkilen, 1982). Such concentrations are of the same magnitude of those found for Ca^{2+} (Torrecilla et al., 2000). As far as we know no data are available on intracellular Co^{2+} concentration, in spite of this cation being part of important proteins, such as cobalamins. In general, few data have been reported on intracellular concentrations of ions in cyanobacteria and on the homeostatic mechanisms to maintain them. With respect to the possible interaction among the different cations tested, future experiments should be performed to assess this possibility, as the resulting data could be important in the study of the regulation of AMDT activity in CYN synthesis.

Acknowledgments

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strain to overexpress AoaA; Dr. Lee Robertson, from the Museo Nacional de Ciencias Naturales for English language help; Dr. Arsenio Villarejo, from the Departamento de Biología UAM, for helping with the purification steps by His-Trap columns; Mª Jesús Vicente, from the SIDI,UAM, for helping with mass spectral analysis; Silvia Juárez and Sergio Ciordia, from the CNB Proteomics Facility, UAM, for their assistance in AoaA sequencing; and José Mª Lázaro, from the CBM, for his comments and advices on subcloning and overexpression.

3.2.6. Complementary work

Initially, to clone and overexpress the *aoaA* gene from *A. ovalisporum* UAM-MAO the GST tagged vector pGEX-KG was used. However, after several time-consuming attempts the expression level was not enough to purify and measure the activity of the resulting protein AoaA. Due to this unsuccessful trial, finally *aoaA* was cloned into pET28b for expression with an N-terminal 6xHis-tag (described in published work of Chapter- II).

3.2.6.1. Cloning aoaA gene into the pGEX-KG expression vector

Amplification of aoaA gene was performed by PCR using the specific primers, *aoaA* forward 5'aaaagaattctaatgcaaacaggaattgtaaatagctgc 3' and *aoaA* reverse 5'aaaaaagcttcaaacctactaaataatgatgaagcg 3', bearing EcoRI and HindIII restriction sites respectively (Fig. 3.2.12).

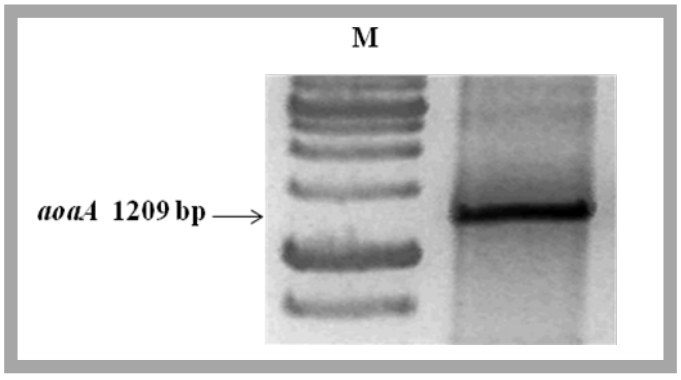

Fig.3.2.12. Specific *aoaA* amplicon for cloning into pGEX-KG vector.

PCR amplification of the cloned *aoaA* gene was carried out in 50 µL of a reaction mixture that contained: 1 µL DNA (100 ng); 1 µL of 10 µM of each primer, 1 µL

dNTPs (25 mM); 1.5 µL $MgCl_2$ (10 mM); 5 µL 10x PCR-Buffer (without $MgCl_2$); 1 µL Taq-DNA-Polymerase (1 U μL^{-1}). All reagents were purchased from Biotools. ThePCR cycle conditions were: 35 cycles (94 ºC for 1 min, 55 ºC for 1 min, 72 ºC for 1 min), final step at 72ºC for 5 min and conservation at 4ºC.

The restriction digestion of the pGEX-KG vector and *aoaA* purified fragment was performed using EcoRI and HindIII Takara Bio Europe restriction enzymes. The insert and vector digestions were cleaned up with kit GFX™ PCR DNA and Gel Band Purification (GE Healthcare), and checked on agarose gel before ligation. The double digestion solution contained: 1-2 µg DNA, 5 µL of 10X Buffer, 10 U EcoRI, 10 U HindIII and water to 50 µL. The mixture was incubated at 37 ºC for 4 h.

The ligation mixture contained two different molar ratios (1:1 and 1:3 insert: vector), 1 µL buffer 10X, 2.5 U of T4 DNA ligase, 0.1 µL of ATP (100 mM) and water to a final volume of 10 µL. The mixture was incubated overnight at 4 ºC. All reagents used were purchased from Takara.

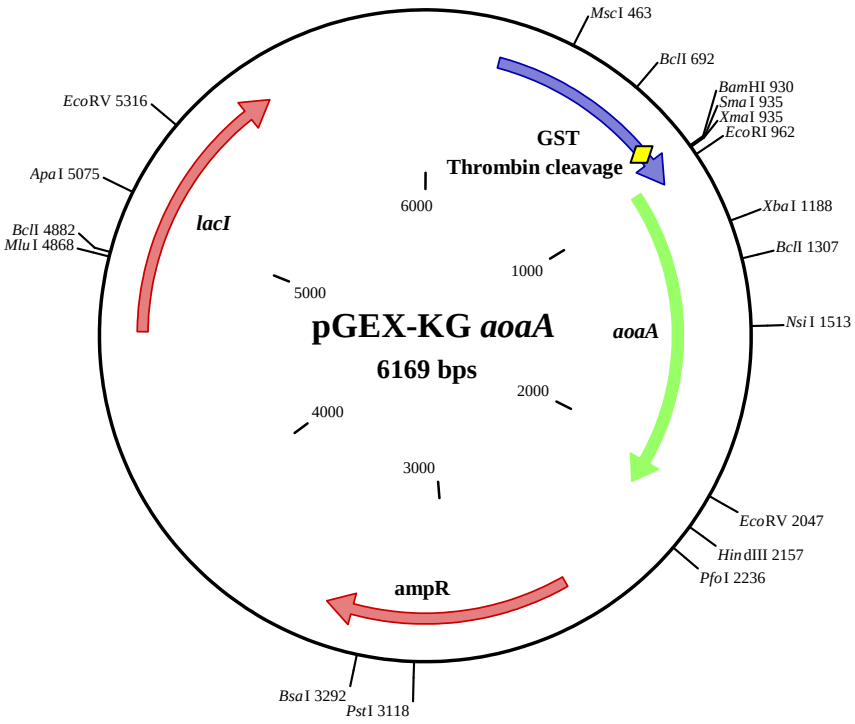

Fig.3.2.13. pGEX-KG vector map and position of the *aoaA* insertion.

The modified plasmid (Fig 3.2.13) was included into *E. coli* BL21 DE3 pLys. Competent cells were prepared and transformed following a calcium chloride protocol (Sambrook and Russell, 2006).

The screening of positive transformants was performed by standar PCR, using bacterial colonies as DNA template (Fig.4.14A) and plasmid digestion with HindIII and EcoRI (Fig.4.14B). The PCR mixture was on a final volume of 50 µL: 1 µL *aoaA* forward primer (10 µM), 1 µL *aoaA* reverse primer (10 µM), 1.5 µL dNTPs (25 mM); 2 µL $MgCl_2$ (10 mM); 5 µL 10x PCR-Buffer (without $MgCl_2$); 1 µL Taq-DNA-Polymerase (1 U μL^{-1}); MilliQ water to 50 µL and 1 bacterial colony was resuspended in the mixture. The PCR conditions were: 35 cycles, 94 ºC for 1 min; 55 ºC for 1 min, 72 ºC for 2 min

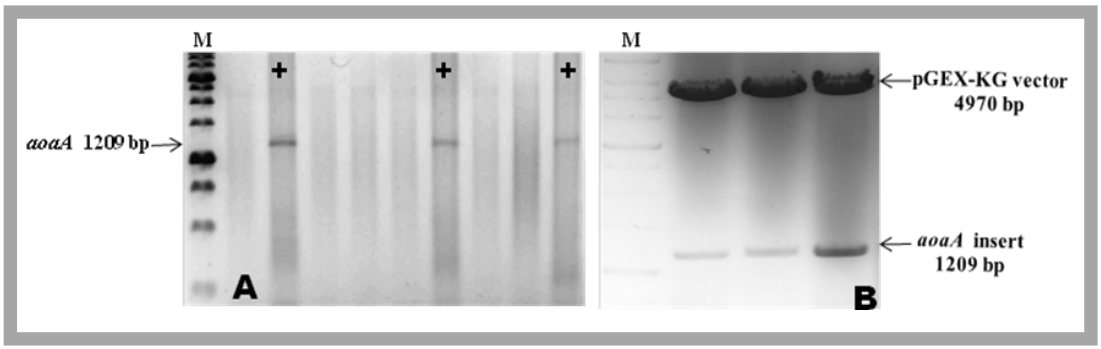

Fig.3.2.14. Screening of transformants. A) Insert amplicons of positive (+) colonies ; B) Plasmid digestion of positive colonies . M, molecular marker.

3.2.6.2. Cell growth, protein overexpression and cell harvesting

The positive transformed colonies were grown at 30 ºC in LB medium with 30 µg mL^{-1} chloramphenicol and 100 µg mL^{-1} ampicillin until reaching an O.D. at 600 nm of 1. GST-fusion protein was induced with 1 mM IPTG for 4h (Fig. 3.2.15 left). Cells were harvested by centrifugation at 5000x g and 4ºC for 20 min.

3.2.6.3. Cell lysis

The cell pellet was resuspended in PBS lysis buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH2PO4 pH 7.4), supplemented with lysozyme (1 mg mL^{-1}, Sigma), Complete™ Protease Inhibitor Cocktail (1 tablet, Roche) 10 mM $MgCl_2$, and DNAse I (10 U mL^{-1}, Roche). Cells were completely lysed by 3 repeated freezing (-

170 °C) and thawing (+30 °C) cycles. The lysate was centrifuged at 50 000 x g for 30 min at 4 °C. The supernatant was used for further purification step of AoaA.

3.2.6.4. Purification of AoaA GST fusion

The supernatant lysate was loaded onto a 5 mL GSTrap FF column.1X PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) was used as binding and washing buffer. 50 mM Tris-HCl and 10 mM reduced glutathione buffer (pH 8) was used for elution.Thrombin (10 U) was added to the eluted fractions containing GST: AoaA protein (Fig. 3.2.15 right (1)) and the mixture incubated at 25 ºC for 5 h for thrombin cleavage (Fig. 3.2.15 right (2)). Thrombin was removed using HiTrap Benzamidine FF columns following manufacturer instructions.

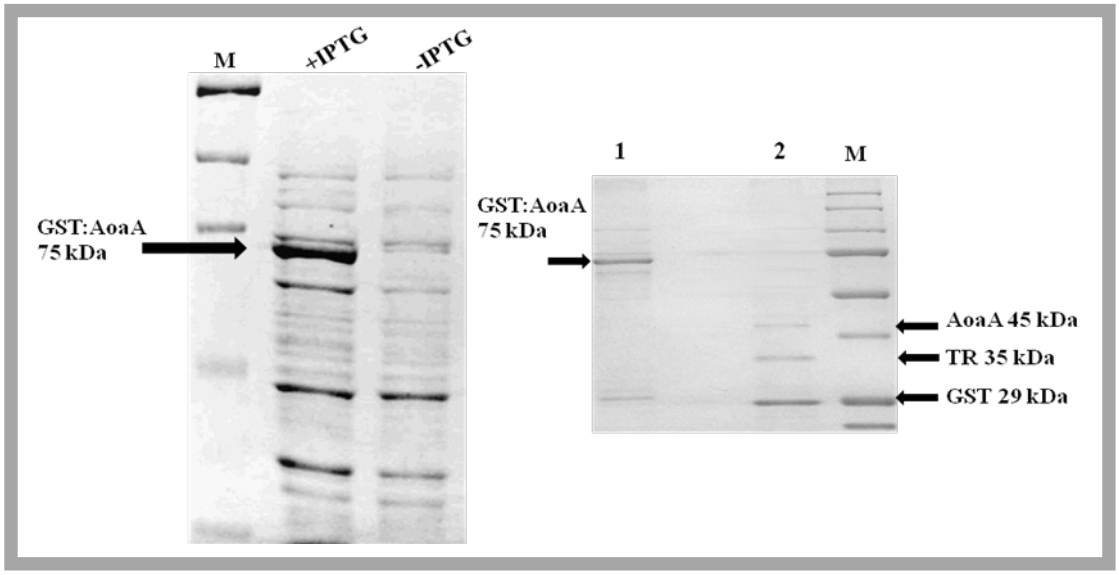

Fig.3.2.15. AoaA recombinant overexpression (left) comparing induced (+IPTG) and non-induced cultures (-IPTG). AoaA purification (right) before (1) and after thrombin cleavage (2). M, molecular weight marker (kDa).

3.2.6.5. Removal of thrombin

The fraction containing AoaA protein and Thrombin (Fig. 3.2.15 right) was loaded into HiTrap Benzamidine FF column previously washed (distilled water) and equilibrated with binding buffer (0.05M Tris-HCl, 0.5 M NaCl, pH 7.4). All fractions were collected until no protein appeared in the efluent (monitored by absorbance at 280nm). The mean yield of the purified AoaA (45 kDa) was 0.1 mg per liter of culture, reaching 90% purity.

3.2.6.6. Prediction of AoaA subcellular location

Subcellular location and function of proteins are closely related, and may reveal interesting features. We have predicted the AoaA subcellular location by several bioinformatic applications; all of them suggesting cytoplasmic location (Table 3.2.2).

Table 3.2.2. AoaA subcellular location.

Prediction method	Subcelullar location	Reference
Gneg-PLoc	Cytoplasm	Chou and Shen, 2008
PSORTb	Cytoplasm	Yu et al., 2010
ClubSub-P	Cytoplasm	Biegert et al.,
Loc Tree	Cytoplasm	Nair and Rost, 2005

3.2.6.7. Putative postranslational modifications

Postranslational modifications are important features for protein function. AoaA sequence was analyzed in silico (De Castro et al. 2006) to find putative patterns of residues able to be modified. Several possible modifications were detected (Table 3.2.3), N-glycosylation (Wang et al., 2013) and Tyrosine kinase phosphorylation (Yang et al., 2013) having been already described in cyanobacteria.

Table 3.2.3. Pattern of putative post translational modifications

postranslational modifications	**Pattern**	**AoaA detected patterns**
N-glycosylation site	N[^P][ST][^P]	217 NRSG
Protein kinase C phosphorylation site	[ST].[RK]	60 TEK 80 TVR 92 SVK 126 SRR 216 TNR 303 SSK 315 SPK
Casein kinase II phosphorylation site	[ST].{2}[DE]	8 SWNE 190 TQDE 247 SHID 291 TSTD 366 TCED
Tyrosine kinase phosphorylation site	[RK].{2,3}[DE].{2,3}Y	130 RFFEYLPY
N-myristoylation site	G[^EDRKHPFYW].{2}[STAGCN][^P]	4 GIVNSW 21 GIADGA 34 GNRPAL 352 GSLHCA

Postranslational modifications described in cyanobacteria are in bold.

3.2.6.8. Predicted secondary AoaA structure

AoaA protein analysis showed a secondary structure similar to that described for amidinotransferases from different origin (Fig. 3.2.16).

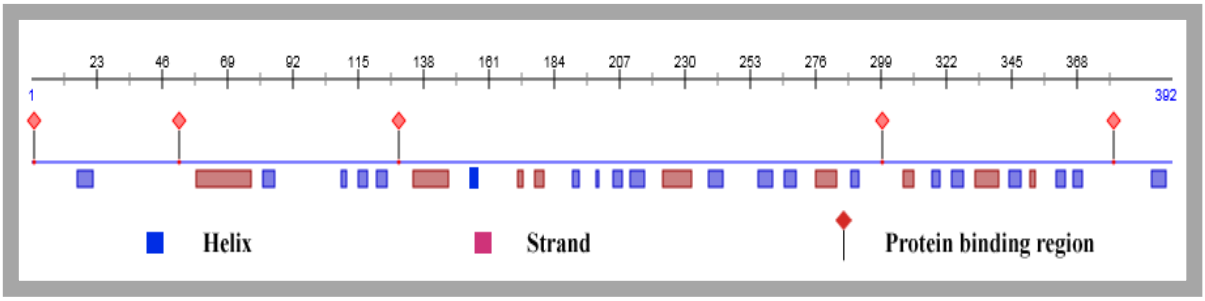

Fig.3.2.16. Predicted secondary structure of AoaA.

3.2.6.9. Amidino group acceptors

Considering the possibility of AoaA accepting other amidino acceptors different from glycine five other related compounds were tested for AoaA cloned into pET28b vector: L-alanine, β-alanine, ethanolamine, L-lysine and α-aminoisobutyric acid. L-arginine was used as amidino donor group, and amidinotransferase activity was determined by the amount of L-ornithine generated. No L-ornithine production was detected with any of the five potential amidino acceptors tested. Therefore glycine appears to be the unique amidino acceptor.

3.3. CHAPTER III

Possible transcriptional control of cylindrospermopsin production in *Aphanizomenon ovalisporum*

Gene expression variation during cylindrospermopsin production in *Aphanizomenon ovalisporum*

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3.3.1. Abstract

Aphanizomenon ovalisporum is a cylindrospermopsin (CYN)-producing cyanobacteria species that due to its increasing worldwide distribution has become an important health alarm in the last few years. Several clusters of genes involved in CYN production have been described in different CYN^+ cyanobacteria genera, named *aoa* for *Aphanizomenon* and *cyr* for *Cylindrospermopsis* and others strains. The sequences of those genes are highly similar, but a rearrangement in gene order is also observed. The information on the control of CYN production by gene expression is still scarce, especially in *Aphanizomenon*. To obtain further information about the control of CYN production in *A. ovalisporum*, we have quantified the intra and extracellular CYN content, during nine days in BG11 batch cultures under optimal conditions. In parallel, the expression of 4 genes related to CYN production, *aoaA-C* and *cyrJ*, has been analyzed by real time q-PCR. The results show a similar pattern of total CYN accumulation and gene expression. Most of the CYN is found intracellularly. Considering the high nitrogen content in the CYN molecule, we have explored if nitrogen assimilation could be related to CYN synthesis. We found inside the *aoaA* and *aoaC* sequences several putative binding domains for the global nitrogen regulator NtcA. The pattern of the *ntcA* expression along the culture is similar to that of CYN accumulation. Our data suggest that CYN production in *A. ovalisporum* seems to be controlled both by the expression of genes *aoa* and *ntcA*, this last one suggesting the influence of available nitrogen, however, other regulation mechanisms of CYN synthesis cannot be discarded.

Keywords: *Aphanizomenon ovalisporum*, *aoa* genes, cylindrospermopsin, *cyr* genes, gene expression, nitrogen and *ntcA*.

3.3.2. Introduction

Aphanizomenon ovalisporum is one of the toxic bloom-forming cyanobacterium in freshwater systems. It shows a potential invasive character due to its high adaptability to different environmental factors (Mehnert et al. 2010; Cires et al. 2011; Sukenik et al. 2012). It seems to be worldwide distributed, having been detected amongst other regions, Australia, Europe, Middle East and the United State. In the last decades, the presence of *A. ovalisporum* has become an important health hazard, because all strains found, with only one exception in Israel (Ballot et al. 2011), produce the toxic alkaloid cylindrospermopsin (CYN).

Other cyanobacteria species have been identified as CYN producers. The gene clusters (*cyr* genes) involved in CYN synthesis have been completely described in *Cylindrospermopsis raciborskii* AWT205 (Mihali et al. 2008), *C. raciborskii* CS-505 (Stucken et al. 2010), *Aphanizomenon* sp. 10E6 (Stuken and Jakobsen 2010), *Aphanizomenon sp.* 22D11 (Stuken and Jakobsen 2010), *Oscillatoria* sp. PCC6506 (Mazmouz et al. 2010) and *Raphidiopsis curvata* CHAB1150 (Jiang et al. 2012). In the case of *Aphanizomenon ovalisporum* (Shalev-Alon et al. 2002) only a partial description of the genes has been reported (*aoa* genes). All *cyr* and *aoa* clusters characterized are highly similar with respect to nucleotide sequences; however, they show several rearrangements in gene order and different flanking regions that might be involved in the expression of *cyr/aoa* genes. Such is the presence of *hyp/hup* sequences in *C.raciborskii* AWT205 (Mihali et al. 2008; Stucken 2010), which could have NtcA binding sites, as in *Nostoc* sp. PCC 73102 (Hansel et al. 2001). Bioinformatic analyses identified putative NtcA binding sites within the *cyr* gene cluster of *C. raciborskii* CS-505 (Stucken 2010). This fact and the high N content in the CYN molecule suggest the influence of N metabolism in the synthesis of the toxin. In general, nutrients seems to modulate CYN production, since nitrogen depletion (Saker and Neilan 2001; Shalev-Malul et al. 2008), phosphate and sulphate starvations caused significant changes in the toxin production (Bacsi et al. 2006).

The model of the CYN biosynthesis pathway (Kellmann et al. 2006; Mihali et al. 2008) includes the activity of an amidinotransferase (AMDT) in the first step, encoded by the *aoaA*/*cyrA* gene. The synthesis proceeds by the action of an enzyme complex constituted by a non-ribosomal peptide synthetase (NRPS) -polyketide synthase (PKS),

codified by the *aoaB*/*cyrB* gene, and further by a PKS encoded by the *aoaC/cyrC* gene. Further PKS activities and other tailoring genes are necessary to complete the synthesis, including the sulfotransferase codified by *cyrJ* gene, and proposed as genetic marker to detect CYN-producing strains (Ballot et al. 2011).

In *A. ovalisporum*, Shalev-Malul and coworkers (2008) identified two transcriptional start points in the *aoaA* and *aoaC* genes, suggesting two modes of regulation of gene expression, one constitutive and the other alternative in response to environmental conditions, such as light intensity and nitrogen depletion (Shalev-Malul et al. 2008), or inorganic phosphate (Pi) deprivation (Bar-Yosef et al. 2010). Moreover, a transcription factor, AbrB-like protein, has been proposed to regulate CYN biosynthesis, its binding region being located between *aoaA* and *aoaC* (Shalev-Malul et al. 2008).

However, in *C. raciborskii* CS-505 transcriptomic analyses of four *cyr* genes (*cyrB*, *cyrI*, *cyrJ* and *cyrK*) under different nitrogen sources have shown almost no variation in gene expression, indicating only a constitutive expression (Stucken 2010). However, different regulation points for individual genes have been observed.

Studies of CYN accumulation under different environmental conditions have been carried out; but limited work has been focused in the relationship between gene expression and toxin production. In addition, the lack of standardization in experimental conditions makes it difficult to draw general conclusions on the possible gene regulation of CYN synthesis. To obtain further insights into the regulation of CYN production in *A. ovalisporum* we have performed BG_{11} batch cultures under optimal conditions, and analysis of the CYN content and the expression of the *aoaA-C genes* were carried out over 9 days. Additionally, the expression of the *cyrJ* gene, a genetic marker of CYN producing strains, and the *ntc*A gene, codifying for the N master regulator, have been tested. Moreover, NtcA putative binding sites inside the *aoa* cluster sequence were searched.

3.3.3. Materials and methods

3.3.3.1. Culture conditions

Aphanizomenon ovalisporum UAM-MAO strain was used throughout the work (Barón-Sola et al. 2012). Three independent experiments were performed with batch cultures in BG_{11} (Rippka et al. 1979), at 28°C, under continuous white light (60 µmole photons m^{-2}

s^{-1}), and continuously bubbling with air passed through a 0.22-µm-pore-size filter. Culture samples were harvested every 24 h during 9 consecutive days. All plots were analyzed in triplicate. Cell observations were done with an Olympus BH-2 microscope at 400x magnification equipped with a Leica DC300F digital system.

3.3.3.2. Growth parameters

Growth was followed both by the optical density at 750 nm ($O.D._{750\ nm}$) and the Chlorophyll *a* (Chl_a) content. Chl_a was extracted in 90% of methanol, and quantified as described Marker et al. (1980).

3.3.3.3. RNA extraction and qPCR

RNA purification was performed using RNeasy Mini Kit Qiagen following manufacturer instructions. Cells were collected from 5 mL of culture by filtration with 0.2 µm Nylon filters. Filters were washed twice with 50 mL of Milli-Q water, and frozen rapidly for RNA extraction. DNAse treatment was applied to RNA samples to remove DNA content and its absence was evaluated performing standard PCR reaction.

RNA quantification was performed by using a Nanodrop® *ND-1000* spectrophotometer.

RNA samples were transcribed to cDNA utilizing random primers p(dN)9 and the high capacity RNA to cDNA Kit (Applied Biosystems).

Based on the genome of *A. ovalisporum* (Shalev-Alon et al. 2002) PCR primers were designed to amplify three *aoa* genes (Table 3.3.1). The sequence of *cyrJ* primers were designed from that of *Aphanizomenon* 10E6 (Stuken and Jakobsen 2010). The *ntcA* primers were devised from *Nostoc* PCC7120 sequences (Kaneko et al. 2001).

Real-time PCR was performed in 10 µL volume including 5 µL Master Mix (SYBR Green, TOYOBO, Japan) and 0,25 µL of each primer (500 nM) **.** The amplification reactions were performed in a AB7.900HT Fast Real Time cycler (Applied Biosystems), under the following conditions: one cycle at 50 ºC for 2 min, one cycle at 95º C for 10 min followed by 40 cycles of 95º C for 15 s, 60º C for 60 s, and 72º C for 30 s. Each reaction was run in triplicate.

Table 3.3.1. qPCR primer sequences

Gene	Function	qPCR Primer sequence (5´-3´)	Amplicon size (nt)
16s rRNA	Ribosomal RNA	q16F CAGTAGCTGGTCTGAGAGGATG q16R GTAGGAGTCTGGGCCGTGT	62
aoaA	AMDT, 1st step CYN biosynthesis	qaoaAF ACCATTCTTTGAGGTAGAGAATCAA qaoaAR AATTTCGTTCCCAAAGGTGA	76
aoaB	NRPS/PKS, 2nd step CYN biosynthesis	qaoaBF TCTCCCCAATAATCTCGCTTAC qaoaBR TGTTCCATCAAGATCCCTTTG	75
aoaC	PKS, 3rd step CYN biosynthesis	qaoaCF GGCACCTGATGGTTGTTGTA qaoaCR CTTCGCCTCGCACATAGC	62
cyrJ	Sulfotransferase, CYN biosynthesis	qcyrJF ATTTCTTTGGGTTGGCGAAT qcyrJR AGACCATGGGAATTGAGTGG	67
ntcA	Transcriptional regulator in N metabolism	qntcAF CGAAACGTTTGAACGCAATA qntcAR AGTAGACTCGTTCGGCAGGA	62

Gene expression data from the qPCR amplification were evaluated using the Ct value, and the 16S rRNA gene was used as control gene to normalize the expression levels of target genes. Relative transcription was calculated using the 2-DDCt method, according to the handbook of Fast Real Time cycler-Applied Biosystems.

3.3.3.4. CYN content determination

CYN content determination was performed from 5 mL in BG_{11} culture, as previously described (Barón-Sola et al. 2012). CYN standard was provided by Abraxis®.

3.3.3.5. Sequence analysis and data representation

Putative NtcA binding domains in the *aoa* cluster (11.35Kb) described by Shalev-Alon et al. (2002) were searched using the CLC sequence viewer® (version 6.8.2). All data analyses were performed using GraphPad Prism® 5.

3.3.4. Results

3.3.4.1. Culture growth

The *A. ovalisporum* UAM-MAO strain was grown in BG_{11} batch cultures, under optimal conditions, during nine days. The growth was checked both by the Chl_a content and the biomass ($O.D._{750}$) (Fig. 3.3.1).

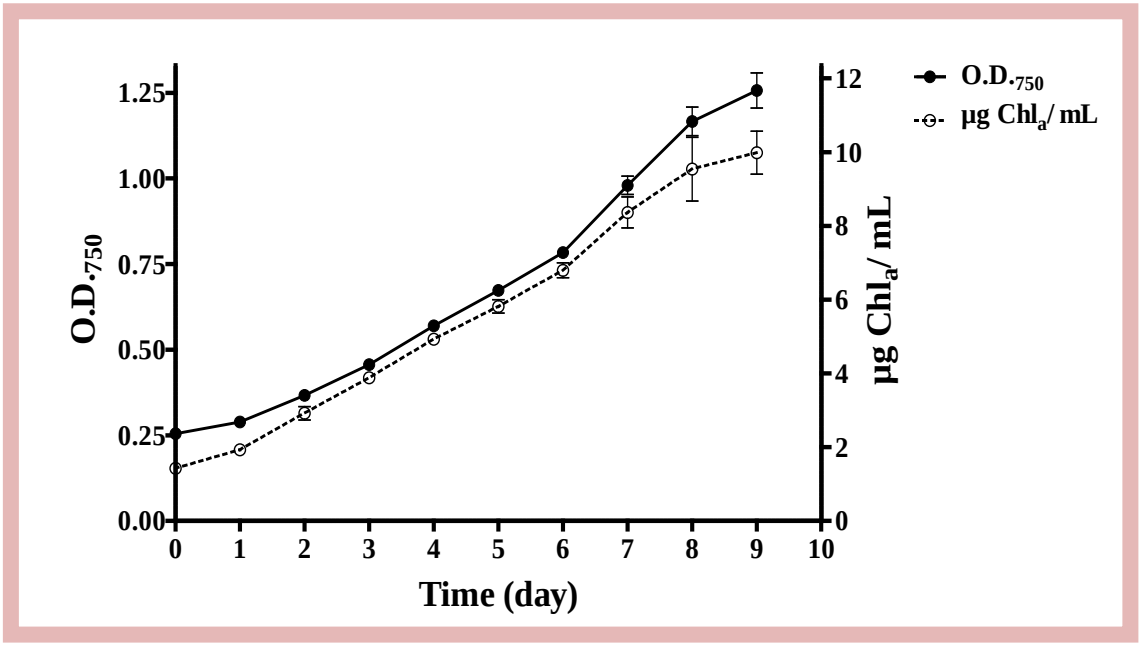


Fig.3.3.1. Growth curve of *Aphanizomenon ovalisporum* UAM-MAO expressed by biomass ($O.D._{750\ nm}$) and Chlorophyll *a*.

The growth pattern after each parameter was different. Thus, the increment of chlorophyll was practically exponential from the start of the culture, and reached its late

exponential phase around the sixth day. However, the biomass exhibited a lag phase, followed by an exponential phase; and late exponential phase was reached at the ninth day. Therefore, only the biomass showed a typical bacterial growth curve. No remarkable morphological changes, neither heterocyst formation, was observed in the filaments along the experiment.

3.3.4.2. CYN production

CYN production was followed by quantifying both internal and external toxin per biomass unit; the pattern of both fractions was quite different. However, the internal and total CYN content had the same tendency, showing three well differentiated phases (Fig. 3.3.2): an increment from the beginning to the 4th day of the experiment, a decrease in the following three days, and a new increment during the two last days.

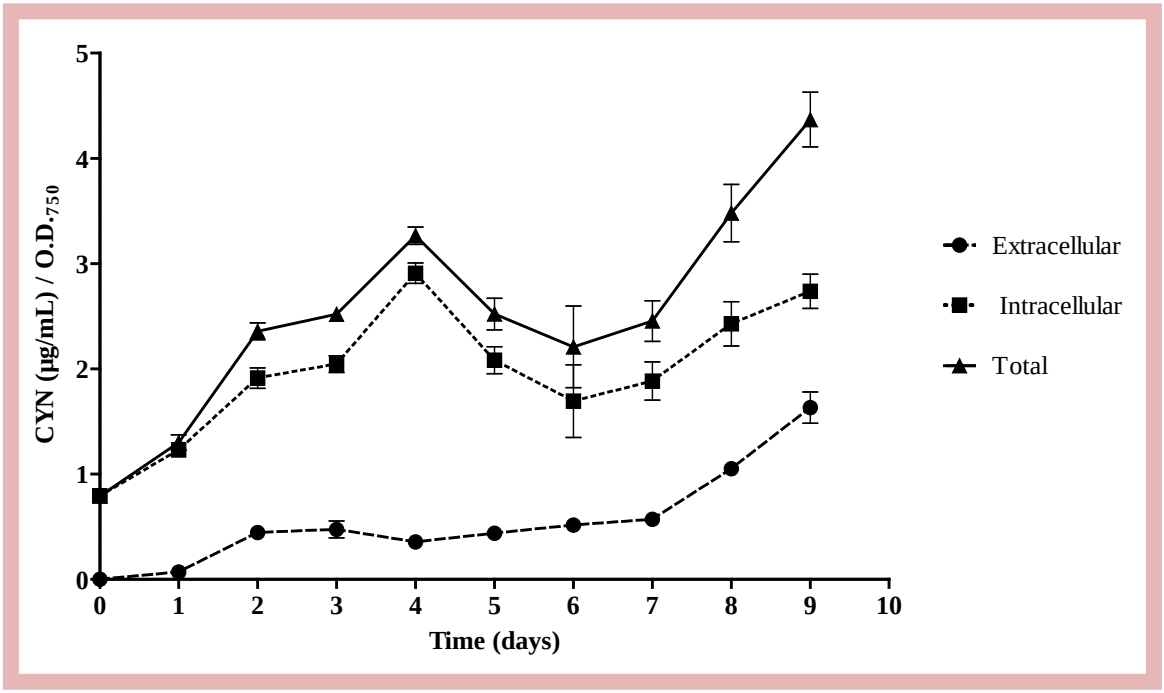


Fig.3.3.2. Cylindrospermopsin accumulation in *A.ovalisporum* UAM-MAO batch cultures. Intra-, extracellular and total CYN content per unit culture volume in function of O.D.$_{750}$ over the time.

It is important to notice that the main contribution to total CYN content throughout the experiment was provided by the intracellular fraction being between 60 to 100%.

3.3.4.3. Gene expression analysis

The kinetics of *aoaA-C* gene expression was studied in parallel with that of CYN accumulation. The expression of the 3 genes showed a similar tendency along the experimental time period (Fig. 3.3.3A-C): an increment between days 2-3, a lag detected during days 4-5, and a further slow recovery in days 6-9, reaching the maximum levels during the last two days. The expression of *aoa*A, codifying for an amidinotransferase, was the earliest detected; and the highest relative expression corresponded to *aoaB* and *aoaC*, encoding NRPK/PKS and PKS enzymes respectively.

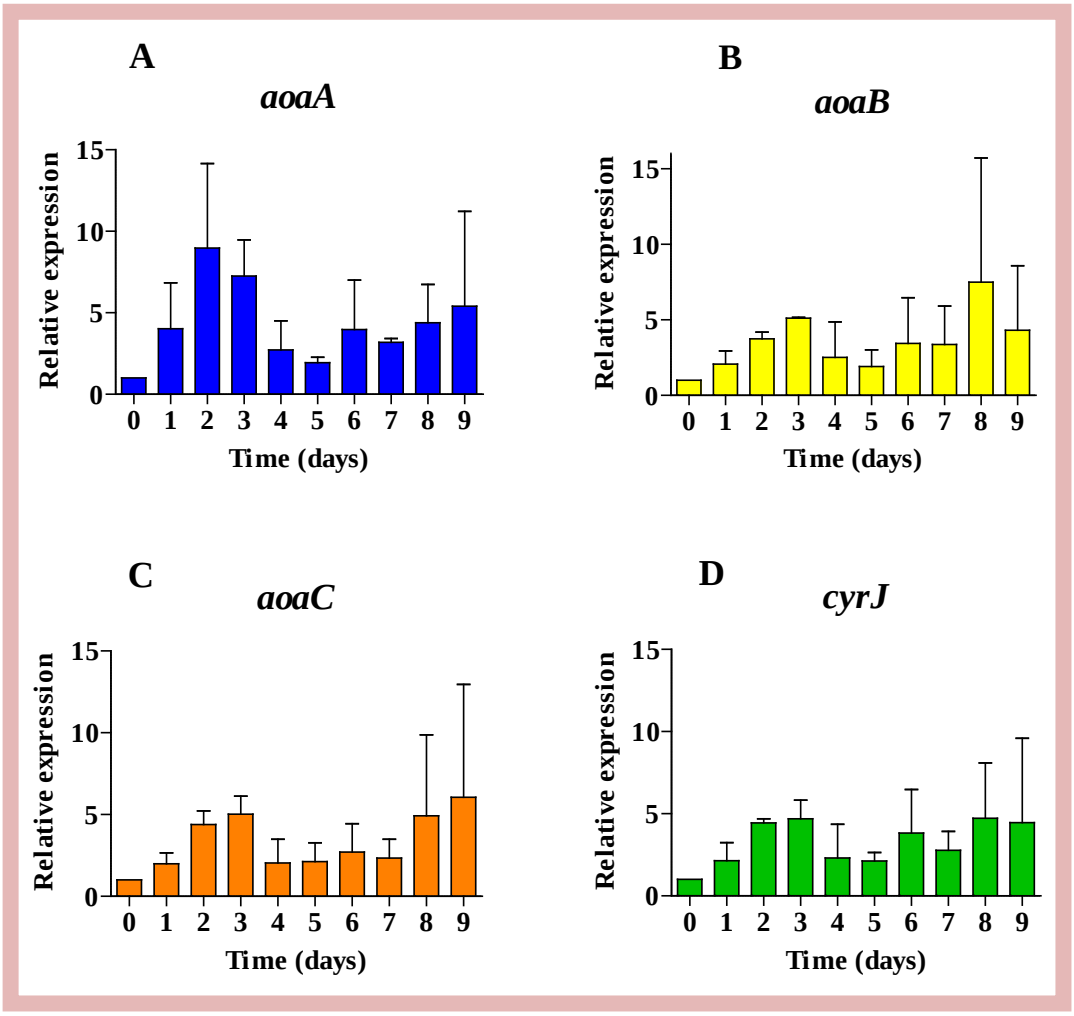


Fig.3.3.3. Expression of genes involved in CYN synthesis along a batch culture of *A. ovalisporum*. Relative expression values (normalized by 16S rRNA), RQ, are given. (A) *aoaA* gene, (B) *aoaB* gene, (C) *aoaC* gene and (D) *cyrJ* gene.

The kinetics of gene *cyrJ* expression was also compared with that of CYN accumulation. In *C. raciborskii*, *cyrJ* encodes a putative sulfotransferase involved in the lasts steps of the CYN biosynthesis pathway. The sequence of the homologous gene in *A. ovalisporum* is unknown; therefore, for the expression analysis we have utilized the sequence of *Aphanizomenon* 10E6. The fluctuations of *cyrJ* transcript accumulation exhibited the same pattern of those of *aoa* genes, reaching maximum levels on day 3 and during days 8-9 (Fig. 3.3.3D).

To study the possible influence of nitrogen metabolism in CYN formation, we analyzed the expression of the *ntc*A gene that that encodes the N-regulator. The pattern of *ntcA* mRNA levels showed a similar trend to that described for *aoaA-C* and *cyrJ* genes (Fig.3.3.4), detecting the highest *ntcA* expression level between the days 8-10.

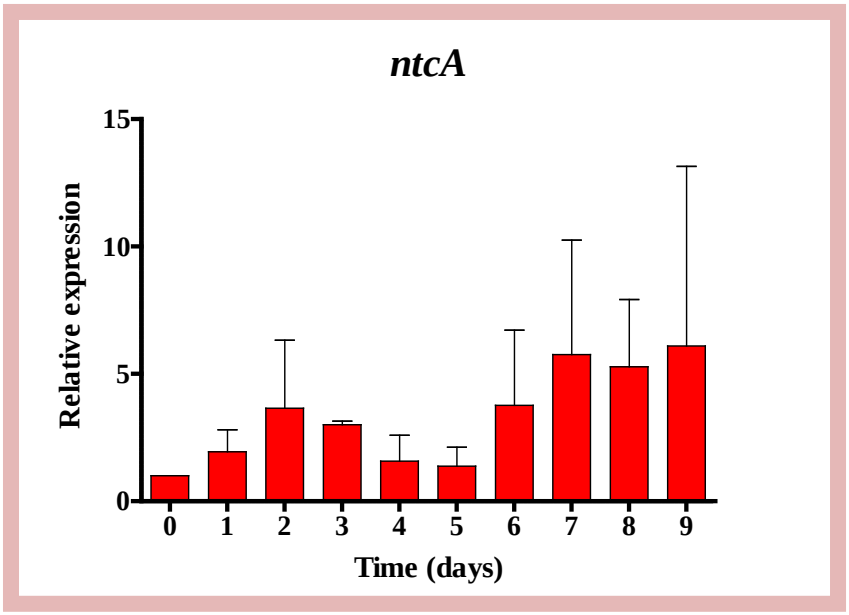


Fig.3.3.4. *ntcA* gene expression analysis along a batch culture of *A. ovalisporum*. Relative expression values (normalized by 16S rRNA), RQ, are given.

By comparing the data of Figs.3.3.2 and 3.3.3, it is apparent that the kinetics of CYN production correlated with that of the relative expression of the genes involved in the CYN synthesis, *aoaA-C and CyrJ*, as well as with that of *ntcA*, related to nitrogen metabolism: the maximum toxin content between days 4-9 fitted with the highest gene expression.

3.3.4.4. NtcA-binding regions

NtcA binding sequences, both canonical and non-canonical types, were searched along the *aoa* cluster (11.35 Kb) described for *A. ovalisporum* by Shalev-Alon et al. (2002). In our search we have also taken into account other putative NtcA binding motifs, as described by Herrero et al. (2001). Three sequences were found inside the *aoa* coding regions (Fig.3.3.5): two were located inside the coding region of the *aoaC* gene, as GTA(N_7)TACN_{23}TAN_3T and GT(N_{10})ACN_{23}TAN_3T sequences; and the third one within the *aoaA* coding region, as GT(N_{10})ACN_{20}TAN_3T. The three sequences agree with those described -10 consensus-like box with the form TAN_3T, separated by 20-24 nt from the putative NtcA binding site upstream (Fig.3.3.5).

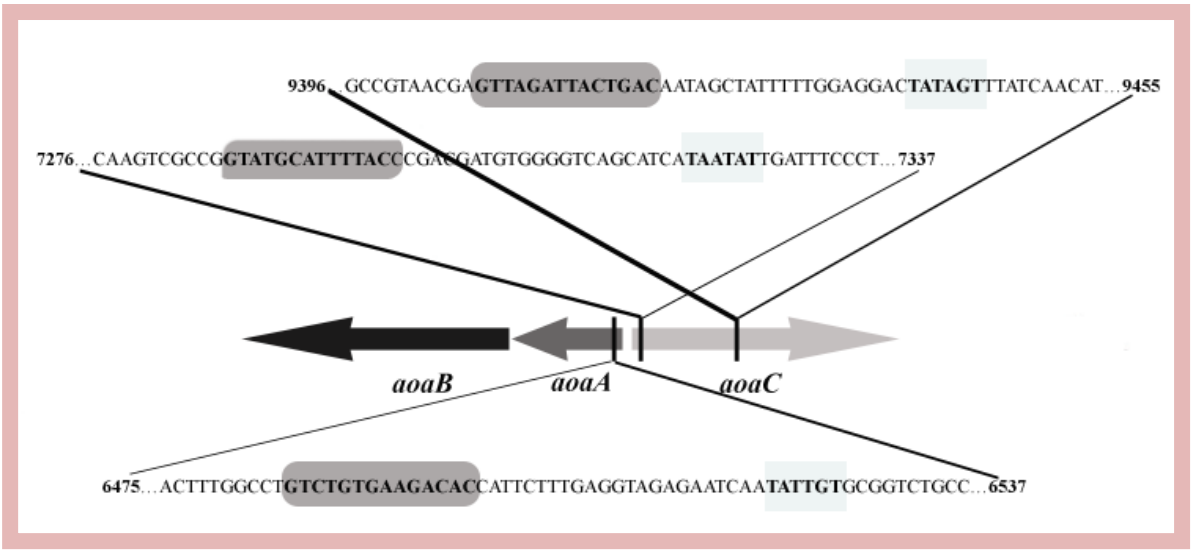


Fig.3.3.5. Putative NtcA boxes within the *aoa* cluster. Putative NtcA binding sites sequences are represented in bold font and enclosed in grey rectangles. Regulatory conserved motif -10 box sequences (TAN_3T), are in bold font and enclosed in light grey rectangles. Location of putative NtcA sequences is marked by a thick black line within the cluster.

3.3.5. Discussion

The final aim of the present work was to contribute to the understanding of the regulation of CYN production in *A. ovalisporum*. We first analyzed the relationship between the CYN production and the expression of four genes considered to be involved in CYN synthesis, *aoaA-C* (Shalev-Alon et al. 2002; Kellmann et al. 2006) and *cyrJ*; and second, we explored if nitrogen metabolism could be related to the control

of the CYN production, by comparing the toxin content and the expression of the nitrogen metabolism control gene *ntcA*, and searching for NtcA-binding sequences in the *aoa* cluster.

The experiments were carried out with batch cultures of the strain of *A. ovalisporum* UAM-MAO (Barón-Sola et al. 2013), grown with nitrate as N source and under other conditions described by other authors as optima for *A. ovalisporum*, temperature (30 ^{o}C; Hadas et al. 2002) and light (60 μmol photons m^{-2} s^{-1}) (Cires et al. 2011). The conditions used seemed appropriate, as indicated by the maximum growth rate attained, 0.24 day^{-1}, similar to the value range previously reported, 0.2-0.36 day^{-1} (Pollingher et al. 1998; Cires et al. 2011;). The calculation of the 0.24 day^{-1} growth rate was based on biomass increase, which under our conditions proved to be a more reliable criterion than chlorophyll. In effect, chlorophyll variation along the culture did not permit us to trace the expected growth pattern of a bacteria culture throughout the time of the experiment (Fig. 3.3.1).

All analytical and gene expression measurements were carried out every 24 h along 9 days, just until late exponential phase of growth to avoid nutrient depletion and the effects of possible inhibitory growth factors. To determine CYN production, both intra and extracellular contents of the toxin were quantified. Intracellular CYN was the main toxin fraction throughout the growth, representing 62.6% to100% of the total toxin content (Fig. 3.3.2). The greater cell CYN content is in accordance with previous studies with cultures of *A. ovalisporum* (Preussel et al. 2009; Cires et al. 2011) and with other data from our laboratory, obtained with another *A. ovalisporum* strain (VAC^{+}) and one of *C. raciborskii* (VCC^{+}), and disagrees with the general idea that in environmental samples CYN appears mainly dissolved in the surrounding medium (Chiswell et al. 1999; Ruecker et al. 2007). Nevertheless, an extracellular location, higher than 40%, could be expected under extreme environmental conditions, during the stationary phase of cyanobacteria growth (Hawkins et al. 2001), and after cell lysis. The existence of CYN+ strains from different species, *A. ovalisporum* included, in which the extracellular CYN were the major toxin fraction cannot be discarding.

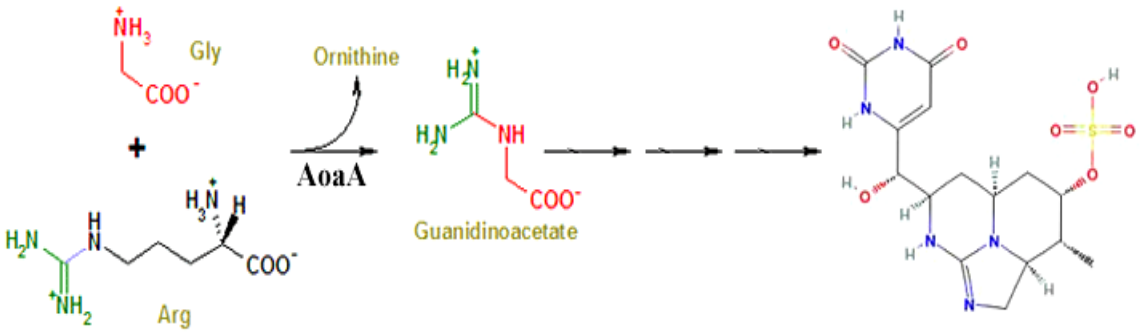
The kinetics pattern of total CYN production correlates with that of *aoaA-C* and *cyrJ* (Fig. 3.3.3) transcript accumulation, suggesting a control of CYN synthesis by those genes. The fluctuations of both CYN and transcript levels were conspicuous at the early

(days 3-4) and late exponential (days 8-9) phase. But in all instances appreciable quantities of CYN and transcripts were observed, indicating a constitutive gene activity. This could justify a constitutive production of CYN in *A. ovalisporum*, as previously suggested (Bacsi et al. 2006; Preussel et al. 2009; Cires et al. 2011). Two transcriptional start points were described for *aoaA* and *aoaC* genes in *A. ovalisporum*, and differential expression levels of those genes were reported under diverse environmental conditions, suggesting the presence of two alternative promoters (Shalev-Malul et al. 2008). The distinct activity of each promoter might account for the fluctuations of both gene transcripts and CYN content.

CYN production as well as the activity of *aoaA*-C and *cyrJ* genes might be modulated by nitrogen metabolism, since the expression pattern of the N-master regulator gene *ntcA* (Fig. 3.3.4) is similar to those of CYN content and the level *aoa*/*cyr* transcripts. Three potential NtcA targets with -10 like box consensus sequence TGT-$N_{9/10}$-ACA, suggested by Ramasubramanian et al. (1994) were located inside the coding region of *aoaA* and *aoaC* genes (Fig. 3.3.5). The putative NtcA sequences are non-canonical, but is not necessarily an impediment to recognize NtcA, since some other sequences have been suggested for NtcA recognition (Herrero et al. 2001; Camargo et al. 2012), which differ from the NtcA sequence GTAN_8TAC reported as the optimal for protein binding. As far as we know, only one canonical intragenic NtcA box has been described in cyanobacteria (Khudyakov and Wolk, 1996). The modulation of CYN synthesis by nitrogen metabolism seems logical, considering the alkaloid nature of the toxin and, therefore the high demand for nitrogen availability. Nitrogen metabolism participation might not be restricted to NtcA; it could well be that a metabolite from nitrogen anabolism or catabolism could also be involved in CYN synthesis, either in the *aoa* gene expression or/and posttranscriptional steps. In any case, further experiments are needed to confirm or discard our suggestions on the modulation of CYN synthesis by either or both NtcA and a nitrogen metabolite.

3.4. CHAPTER IV

First evidence of accumulation of guanidinoacetate in cyanobacteria



Cylindrospermopsin

First evidence of accumulation of guanidinoacetate in cyanobacteria

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Manuscript submitted

3.4.1. Abstract

Guanidinoacetate (GAA) is one of the most extensively studied toxic guanidine compounds. Changes in GAA can affect the nervous system and induce hyperhomocysteinemia, representing a risk factor for cardiovascular diseases. In cyanobacteria, GAA is thought to be an intermediate in the synthesis of the toxin cylindrospermopsin (CYN), one of the most common known cyanotoxins that affects multiple organs and functions in animals and plants. In spite of the evidence supporting GAA toxicity and its role in CYN synthesis, no data have been reported on the accumulation of GAA in any cyanobacterium. We have analyzed and compared the content of GAA in cultures of diverse cyanobacteria types, both cylindrospermopsin producing (CYN^+) and not producing (CYN^-). The results obtained show that GAA accumulates in the majority of the strains tested, although the highest content was found in one of the CYN^+ strain, *Aphanizomenon ovalisporum* UAM-MAO. In this strain, both GAA and CYN can be located within and out the cells. In conclusion, GAA appears to be a general cyanobacterial metabolite that due to its proven toxic should be considered when studying cyanobacterial toxicity, and in cyanotoxicity management.

Keywords: Guanidinoacetate, cylindrospermopsin, guanidino compounds, cyanobacteria toxicity, *Aphanizomenon ovalisporum*

3.4.2. Introduction

An ample range of organisms are able to synthesize highly water-soluble guanidino compounds (Berlinck et al., 2012), some of which have been described as toxic (Table

3.4.1). Guanidinoacetate (GAA) is one of the most extensively studied guanidine derivatives, due to its pivotal role in energy metabolism, by acting as a creatine precursor. Metabolic disorders, such as defects in guanidinoacetate-methyltransferase (Gordon, 2010) or creatine transporter (Braissant et al., 2011) lead to the accumulation of GAA in the brain, blood, cerebrospinal fluid and urine. GAA toxicity in humans is mainly attributed to the inhibition of acethylcholinesterase (Delwing-de Lima et al., 2010) and Na+, K+ -ATPase activity (Zugno et al., 2006), and increased production of reactive oxygen species in different tissues (Mori et al., 1996; Zugno et al., 2008). These alterations affect predominantly the nervous system, where gabaergic neurotransmissions, glutamate uptake and acethylcholinesterase activity can be severely compromised (Neu et al., 2002; Zugno et al., 2007; Spanevello et al., 2008). Moreover, GAA induces an increase of homocysteine concentration in plasma (hyperhomocysteinemia), representing a risk factor for cardiovascular diseases (Stead et al., 2001; Fukada et al., 2006; Setoue et al., 2008; Williams and Schalinske, 2010; Liu et al., 2011).

Table 3.4.1. Examples of toxic guanidine compounds

Compound	Toxicity mechanism	Reference
Guanidinoacetic acid	Inhibition of: Na^+K^+-ATPase Creatine kinase Acetylcholinesterase Glutamate uptake GABA A receptor activation ROS production estimulation	Spanevello et al., 2008 Zugno et al., 2006, 2007, 2008 Neu et al., 2002 Cupello et al., 2008 Mori et al., 1996
α-guanidinoglutaric acid	Free radical generation Nitric oxid synthase inhibition	Mori et al., 1995 Yokoi et al., 1994
Guanidinopropionic acid	Inhibition of: Glucose 6 phosphate dehydrogenase Creatine uptake	Oudman et al., 2013
Homoarginine	Butyrylcholinesterase inhibition	Hiramatsu et al., 2003; Delwing-de lima et al., 2010
Methylguanidine	GABA A receptor inhibition	De Deyn et al.,1990
L-Canavanine	Misincorporation of amino acid analogs into protein	Bence and Crooks, 2003
Guanidinosuccinic acid	Inhibition of: GABA A receptor Platelet factor III activation	De Deyn et al., 2009
Saxitoxin and analogs	Sodium channel blockage	Wiese et al., 2010
Cylindrospermopsin	Protein synthesis inhibition	Terao et al., 1994; Froscio et al., 2007

In cyanobacteria, guanidine derivatives are thought to act as intermediates in the synthesis of the toxins cylindrospermopsin (CYN) (Kellmann et al., 2006) and paralytic shellfish poisoning compounds (Kellmann et al., 2008) (Fig. 3.4.1). CYN is after microcystins (MCs) the most frequent cyanotoxin reported so far (Falconer and Humpage, 2006), and its occurrence and distribution are increasing world-wide (Kinnear, 2010). It is a tricyclic guanidine alkaloid produced by several cyanobacteria species, and is known as a potent cytotoxin that affects multiple organs and functions in

animals (Froscio et al., 2009) and plants (Beyer et al., 2009), causing fatalities in cattle and outbreaks of human illness (Griffiths and Saker, 2003; Bownik, 2010). The action of CYN has been mainly described in liver and kidney cells, but other cell types, such as neurons can also be affected (Kiss et al., 2002).

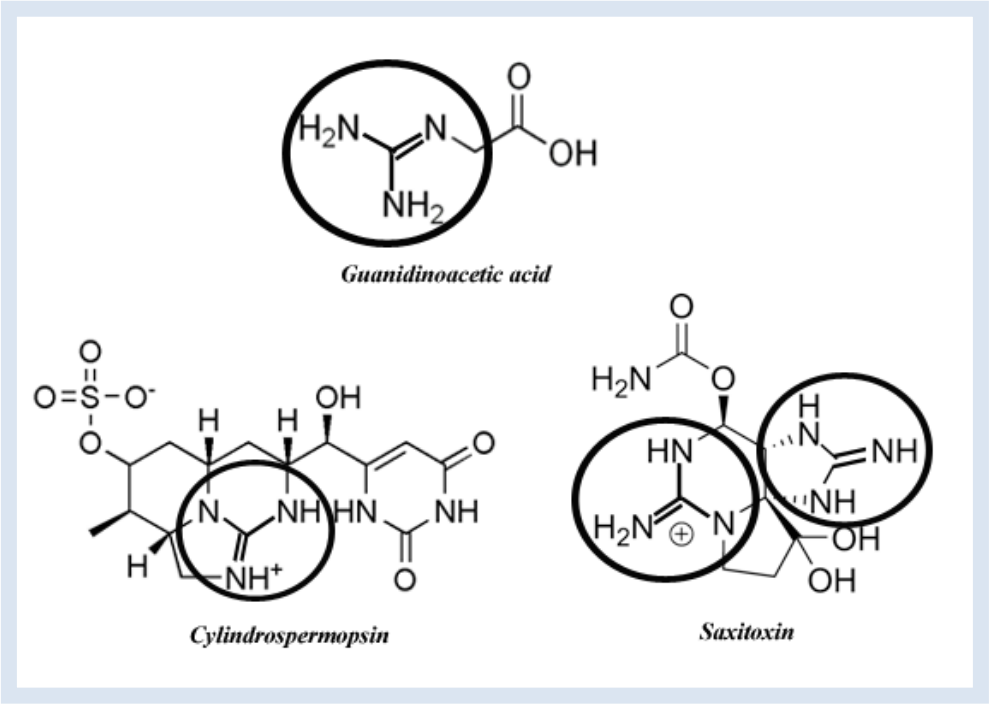

Fig.3.4.1. Examples of guanidine compounds synthesized by cyanobacteria.

Although the synthesis of CYN is not yet well established, there are genetic and biochemical evidence that GAA is formed in the first step of the pathway, due to the activity of an amidinotransferase. This enzyme specifically utilizes arginine and glycine as an amidino group donor and acceptor, respectively (Muenchhoff et al., 2010; Barón-Sola et al. 2013). Further steps would involve the participation of diverse enzymes, such as a polyketide synthase and a non-ribosomal peptide synthetase (Mihali et al., 2008).

In spite of the evidence supporting GAA toxicity and its role in CYN synthesis, no data have been reported on the accumulation of GAA in CYN^+ cyanobacteria, or even in any cyanobacterium. This lack of information moved us to analyze and compare the content of GAA in cultures of diverse cyanobacteria types, both CYN^+ and CYN^-. The results obtained show that GAA accumulates in all the strains studied. Therefore, GAA appears to be a general cyanobacterial metabolite. We think that these findings, along with the

proven toxic effects of GAA, would justify that this compound be considered when studying cyanobacterial toxicity.

3.4.3. Materials and methods

3.4.3.1. Bacteria strains and culture conditions

Eleven cyanobacteria strains of different types were used (Table 3.4.2): unicellular, filamentous, CYN^+, CYN^-, MC^+ and MC^-. All were cultured in BG_{11} medium (Rippka et al., 1979) with constant aeration, under continuous fluorescence light (60 µmol m^{-2} s^{-1} PAR), at 28 ºC. The *Escherichia coli* strain BL21 (DE3) pLys, used as a GAA negative control, was cultured in LB medium at 37 ^oC.

Table 3.4.2. Bacteria utilized in the present work

Strain	Known cyanotoxin
***Aphanizomenon ovalisporum* UAM-MAO**	CYN
***Cylindrospermopsis raciborskii* VCC+**	CYN
***Aphanizomenon ovalisporum* VAC+**	CYN
***Cylindrospermopsis raciborskii* VCC-**	nd
***Microcystis aeruginosa* UTEX 2385**	MC
***Microcystis aeruginosa* UTEX 2666**	MC
***Microcystis aeruginosa* PCC 7806**	MC
***Microcystis aeruginosa* UAM-CP101**	nd
***Microcystis flos-aquae* UTEX 2677**	nd
***Anabaena* sp. PCC 7120**	nd
***Nostoc* sp. UAM-N1**	nd
***Escherichia coli* BL21 (DE3) pLys**	nd

nd= not detected; UTEX and PCC strains were obtained from the University of Texas collection and Pasteur culture collection respectively. *C. raciborskii* VCC^+ and VCC^- as well as *A. ovalisporum* VAC^+ were kindly provided by Dr.Vasconcelos, from the University of Porto, Portugal. UAM strains were isolated in our laboratory. *E. coli* strain was included as a negative GAA control.

3.4.3.2. Analysis of guanidinocetate and cylindrospermopsin

Intracellular GAA and CYN, as well as dissolved extracellular CYN were directly quantified in samples taken every 24 h during 9 days. Extracellular GAA in the cultures could not be directly determined, due to the interference of the culture medium salts in the analysis; however, dissolved GAA could be quantified after discarding the culture medium by centrifugation and resuspending the cells in water.

For direct intracellular GAA quantification, 4 mL of culture were removed, centrifuged, the pellet lyophilized, and suspended in 1 mL of sterile Milli-Q water and stored at -20 °C for further analysis. To compare intracellular and extracellular GAA, 10 mL culture was centrifuged (5000 x*g* 10 minutes) and the sedimented cells washed and finally resuspended in 5 mL sterile Milli-Q water. After incubation under the same conditions used for culture, the samples were centrifuged. GAA was determined in cells and supernatant, to determine intracellular and extracellular content, respectively. GAA was analyzed by HPLC – electrospray mass spectrometry (HPLC-ESI-MS) using a Varian 1200 L triple-quadrupole mass spectrometer, with an ESI. The chromatographic method was carried out on a Synergi 4u, Polar RP, 80A, 150 mm x 4.6 mm (Phenomenex) column, using a mobile-phase gradient (Table 3.4.3).

Table 3.4.3. Chromatographic gradient conditions used for GAA determination

Time (min)	%A: Methanol	% B: 10 mM Ammonim acetate in Milli-Q water	Flow (mL/min)
0:00	10	90	0.2
15:00	60	40	
16:00	90	10	
20:00	90	10	
21:00	10	90	
30:00	10	90	

ESI-MS determinations were performed in the positive ionization mode, under the following conditions: needle voltage, +5 kV; shield voltage, 0.6 kV; nebulizing gas (N_2)

pressure, 50 psi; drying gas temperature, 250 °C; drying gas pressure, 25 psi; ion source temperature, 50 °C, and capillary voltage, 40V. ESI-MS spectra were obtained by introducing 5 µL into the system. GAA was determined by quantifying the product ion 72 *m/z* (Figure 3.4.2).

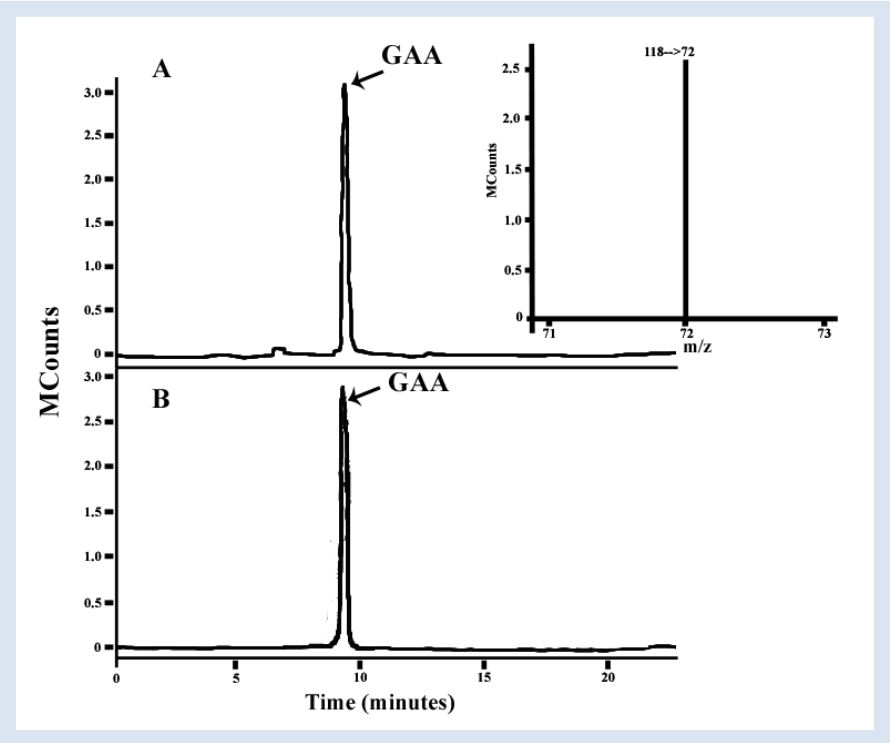

Fig.3.4.2. HPLC chromatogram and MS fragmentation of GAA. A) Chromatogram of *Aphanizomenon ovalisporum* UAM-MAO crude extract; B) GAA standard. Insert in A, detail MS fragmentation pattern of GAA.

A calibration curve (0.1- 30 µg mL^{-1}) with standard GAA (Sigma-Aldrich®) in 10 mM ammonium acetate was constructed to calculate the concentration in the samples. The detection limit was 0.01µg mL^{-1}.

CYN was routinely determined both in cells and in the medium. To separate the two fractions, 15 mL of culture were vacuum-filtered (Whatman® GF/F filters 0.2 µm). The cell content was extracted by sonication in 10 mL of 5% formic acid. After centrifugation, the supernatant was concentrated in a rotary evaporator and suspended in 1 mL sterile Milli-Q water for further analysis. The dissolved CYN fraction was frozen, lyophilized and suspended in 1.5 mL of sterile Milli-Q water. Quantification of CYN in both fractions was performed using the HPLC-DAD system, following the method of Törökné et al. (2004). A calibration curve with standard CYN (Abraxis®) was used for quantification.

3.4.4. Results

3.4.4.1. Accumulation of GAA in different types of cyanobacteria

To explore if GAA could be accumulated in cyanobacteria, we first analyzed its content in cells of 3 CYN^+ strains (two of *A. ovalisporum* and one of *C. raciborskii*).

Table 3.4.4. Cell guanidinoacetic acid content in different bacterial strains

Strain	GAA (μg mg^{-1} dry weight)
***Aphanizomenon ovalisporum* UAM-MAO**	**1.35[a]**
***Cylindrospermopsis raciborskii* VCC+**	0.09
***Aphanizomenon ovalisporum* VAC+**	**0.31**
***Cylindrospermopsis raciborskii* VCC-**	**0.15**
***Microcystis aeruginosa* UTEX 2385**	**0.68**
***Microcystis aeruginosa* UTEX 2666**	0.03
***Microcystis aeruginosa* PCC 7806**	0.02
***Microcystis aeruginosa* UAM-CP101**	0.04
***Microcystis flos-aquae* UTEX 2677**	**0.21**
***Anabaena* sp. PCC 7120**	0.02
***Nostoc* sp. UAM-N1**	0.01
***Escherichia coli BL21* (DE3) pLys**	0.00

a. Bold face: higher values

It was found that GAA accumulated significantly in the two strains of *A. ovalisporum*, especially in UAM-MAO, but not in *C. raciborskii* (Table 3.4.4). Later, GAA was analyzed in strains of diverse types, all of them CYN^-: unicellular, filamentous, microcystin-producing (MC^+) and non-producing (MC^-). MC^+ strains were included due to the well-known toxicity of MCs. The results obtained (Table 3.4.4) show that the MC^+ *M. aeruginosa* UTEX2385 and the CYN^- MC^- *M. flos-aquae* UTEX2677 strains accumulated GAA to an extent comparable to that of *A. ovalisporum.* Therefore, the CYN^+ character does not appear to be an important factor for GAA accumulation.

3.4.4.2. Kinetics of accumulation and release of CYN and GAA

Further experiments were carried out to determine the capacity of cells to accumulate and release GAA and CYN. For that, we first determined the cell content of GAA and CYN along a 9 day culture of *A. ovalisporum* UAM-MAO (Fig. 6.3).

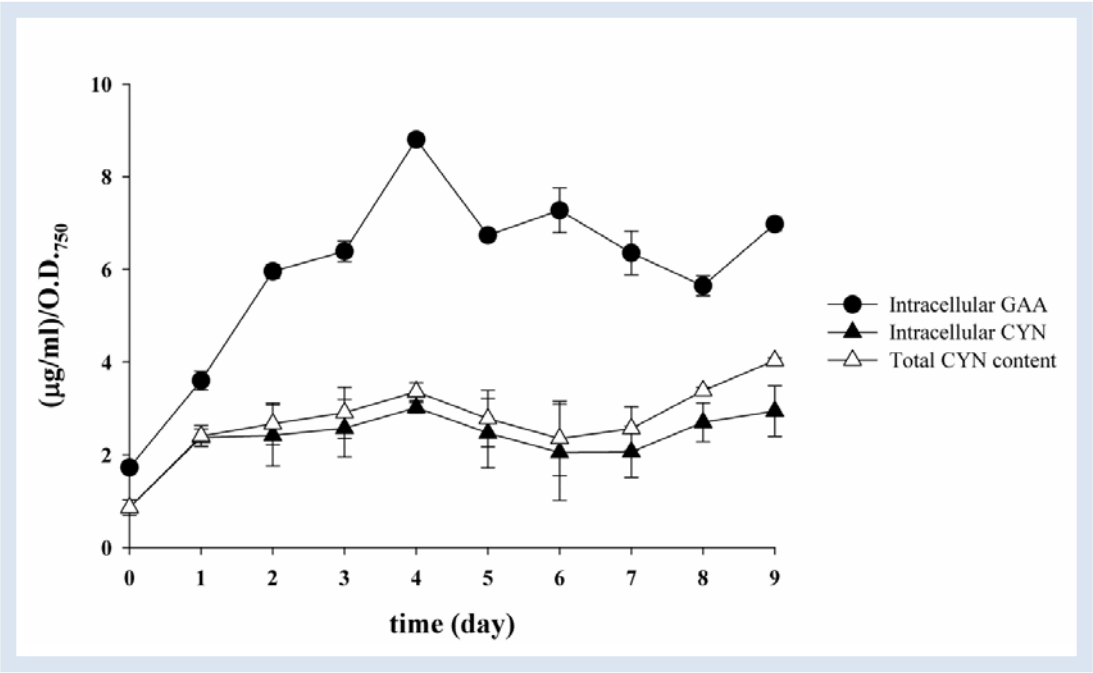

Fig.3.4.3. Intracellular GAA content (dark circles), intracellular CYN (dark triangles) and total CYN content (open triangles) in cultured cells along the growth of the CYN^+ strain *Aphanizomenon ovalisporum* UAM-MAO. Each point represents the mean ± SD (n=3).

The data are given in relation to biomass (O.D. $_{750}$), to express the cells capacity to accumulate the two compounds. The accumulation profile of GAA and CYN was similar: they increased from the beginning of the culture, and attained a steady state after 2-3 days that lasted until at least the beginning of the stationary phase (about 9 days). GAA content was always higher than that of CYN at all growth stages, especially during the exponential phase (2^{nd} -9^{th} day) where GAA concentration was 2-4fold than that of CYN. Figure 3.4.3 also shows the variation of total (intracellular and extracellular) CYN. Extracellular CYN would allow inferring the cell capacity to extrude the toxin. In all samples most CYN was mainly located within the cells (more than 80%); but the percentage of extracellular CYN slightly increased in the late phase of growth. Extracellular GAA, as indicated in the Methods Section, could not be directly quantified; nevertheless, after removal of the culture medium the released GAA from cells was analyzed.

Table 3.4.5. Release of GAA from *A. ovalisporum* UAM-MAO cells

GAA assay time (h)	GAA (μg mL^{-1})			
	O.D.$_{750}$=0.6		O.D.$_{750}$=1.2	
	Intracellular	Extracellular	Intracellular	Extracellular
0	5.3±0.2	-	4.7±0.1	
24	6.7±0.05	1.1±0.1 (14%)	4.8±0.2	3.1±0.15 (39%)
48	-	-	5.2±0.1	0.55±0.1 (10%)

Table 3.4.5 shows the values of the initial intracellular GAA, as well as the extracellular GAA after 24 and/or 48 h after removal of the medium. Two cultures at different growth phase (O.D.$_{750}$, 0.6 and 1.2) were used. During the assay points, the percentage of extracellular GAA was in all cases larger than the intracellular percentage (Table 3.4.5). Nonetheless, extracellular GAA in the older culture (O.D. 1.2) was significantly high (39%) after 24 h. Curiously, this percentage was much lower after 48 h. As a whole, it appears that GAA can indeed be released from the cells and that the exit capacity might depend on the cell status.

3.4.5. Discussion

The results show that GAA is accumulated in cyanobacteria of diverse types (Table 3.4.4). As far as we know this is the first time that GAA has been quantified in cyanobacteria. Being GAA an intermediate metabolite in CYN synthesis (Mihali et al., 2008; Muenchhoff et al., 2010; Barón-Sola et al., 2013) it could be expected that its accumulation might be higher in CYN$^+$ cyanobacteria. Indeed, the CYN$^+$ strains tested accumulated GAA, the largest cell concentration being observed in *A. ovalisporum* UAM-MAO (Table 3.4.4); but, there appears to exist no relationship between the CYN$^-$ producing capacity and GAA content (Table 3.4.4), since in some of the CYN$^-$ strains, as MC$^+$*M. aeruginosa* UTEX2385, GAA level was similar and even higher than that of two CYN$^+$ strains. On the other hand, GAA accumulation does not appear to be related to MC production. Taking together, our data suggest that in cyanobacteria, GAA is a metabolite that could be involved in metabolic pathways different from those related to the so-far known cyanotoxins. It would be interesting to explore the presence of AMDTs or other enzymes that could account for GAA production in cyanobacteria, as

the reversible guanidinoacetate amidinohydrolase described in fungi (Brunel et al., 1969) and bacteria (Yorifuji et al., 1977). In this respect, the genomes of quite a few CYN^- cyanobacteria, unicellular and pluricellular show AMDT sequences different from those related to CYN synthesis (GenBank® Database). Even in the case of CYN^+ strains, it is possible the existence of AMDTs different from that involved in CYN synthesis.

To date, the toxicity of cyanobacteria has been mainly attributed to few toxins, including MCs, CYN, saxitoxin, anatoxin-a, and lipopolysacharides. Therefore, only such toxins are regularly analyzed to infer the toxicity and ecological risk of cyanobacteria. However, it has been reported that the toxicity attained by crude extracts from different cyanobacteria with or without the most commonly known toxins, such as MCs (Jungmann, 1992; Palikova et al., 2007; Puerto et al., 2010) or CYN (Hawkins et al., 1997; Falconer et al., 1999; Humpage and Falconer, 2003) was higher than that caused by the purified toxins (Ohtani, et al. 1992; Kiss et al., 2002; Gutiérrez-Praena et al., 2012), indicating the presence of other toxic compounds with additive and/or synergistic effects in the extracts. GAA, whose toxicity has been widely studied in humans (Table 3.4.1), appears to be a good candidate to justify at least partially that extra toxicity. Our results, along with the numerous evidence of the deleterious effects of GAA in humans, strongly suggests the possibility that GAA contributes to cyanotoxicity, and could be one cause of the higher toxicity in crude cyanobacteria extracts when compared to the known toxins alone. The synergistic effects would undoubtedly enhance the health risk in cyanobacteria with CYN, MC and other known toxins. Those effects could be extended to other type of organisms, namely plants, where the inhibition of oxygen uptake in mitochondria by GAA has been reported (Durzan, 2010).

Our data refer mainly to intracellular GAA; but, it was established that GAA is able, as CYN (Fig. 3.4.3), to exit from cells (Table 3.4.5). That suggests that in cyanobacteria blooms, GAA might be present both in the cyanobacteria and in the surrounding water. At any rate, we think that GAA should be considered in the assessment and management of water cyanotoxicity.

It may well be that other toxic guanidine compounds, besides CYN, PSPs and GAA, are present in cyanobacteria, being able to contribute to cyanotoxicity. Future studies should be aimed to: i) investigate whether any toxic guanidine compounds are

accumulated in planktonic cyanobacteria; ii) analyze in different types of organisms the synergistic and/or antagonistic toxic effects of GAA with other components of cyanobacteria extracts, including known cyanotoxins. Lastly, it would be interesting to amplify the scope of studies on guanidine compounds, by analyzing their presence in food supplements derived from algae or cyanobacteria, where cyanotoxins, such as CYN, have been detected (Liu and Scott, 2011).

Acknowledgments

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3.4.6. Complementary work

Zebrafish embryo is an aquatic animal model system used to search the effect of toxic agents in developmental stages. This system has been applied to investigate microbial toxins in water environmental. Here, we used this system to check the toxic effect of GAA, since it might be accumulated in freshwater blooms together with the rest of cyanotoxins.

3.4.6.1. GAA toxicity in zebrafish (Danio rerio)

Two types of toxicity assays were performed: in one of them, whole and dechorinated embryos were immersed in GAA solutions; in the other one, GAA was microinjected into whole embryos.

In the first test, embryos (4-to 32-cell stage) were collected within 2 hours post-fertilization (hpf) and exposed to GAA, by dipping during 5 days in wells of 24-well polypropylene plates (25 embryos per well) containing different GAA (Sigma-Aldrich®) concentrations (2, 20 and 200 µM) in either E3 medium or distilled water. The assays were done in triplicate. Morphological changes were observed under light microscope every 24 h. Neither toxic effects on zebrafish development nor significant mortality respect to the control were observed (Fig.3.4.4).

Fig.3.4.4.Untreated zebrafish embryos at 24 h pf (A) and 48 h pf (B).

In the microinjection test, fertilized eggs were microinjected with ~4 nL of different GAA solutions to reach the same GAA final embryo concentrations used in the immersion test, 2, 20 and 200 µM. To calculate the final GAA concentration, it was assumed that the embryo volume was 0.2 µL. The effects were observed 24 h after microinjection (pmi). The assays were performed in duplicate. Unlike immersion, microinjection caused the death and important abnormalities in the embryo development (Fig.3.4.5), such as pericardial edema, impairment of eye formation and diverse morphological abnormalities in head and trunk. The death effect was dose-dependent. But, in the development changes, the data obtained so far did not permit to get a well-defined toxicity pattern in relation to the GAA doses assayed (Fig.3.4.6).

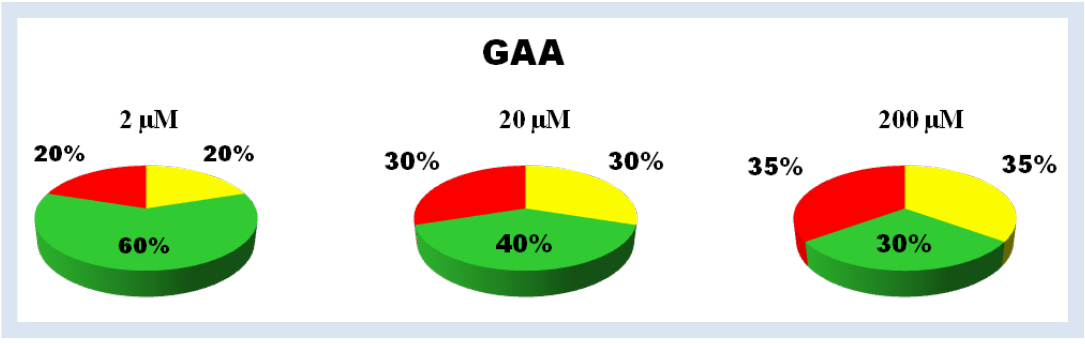

Fig.3.4.5. Effect of different GAA concentrations on zebrafish embryos at 24 h pmi. Unaffected (green), abnormal (red) and dead (yellow) embryos.

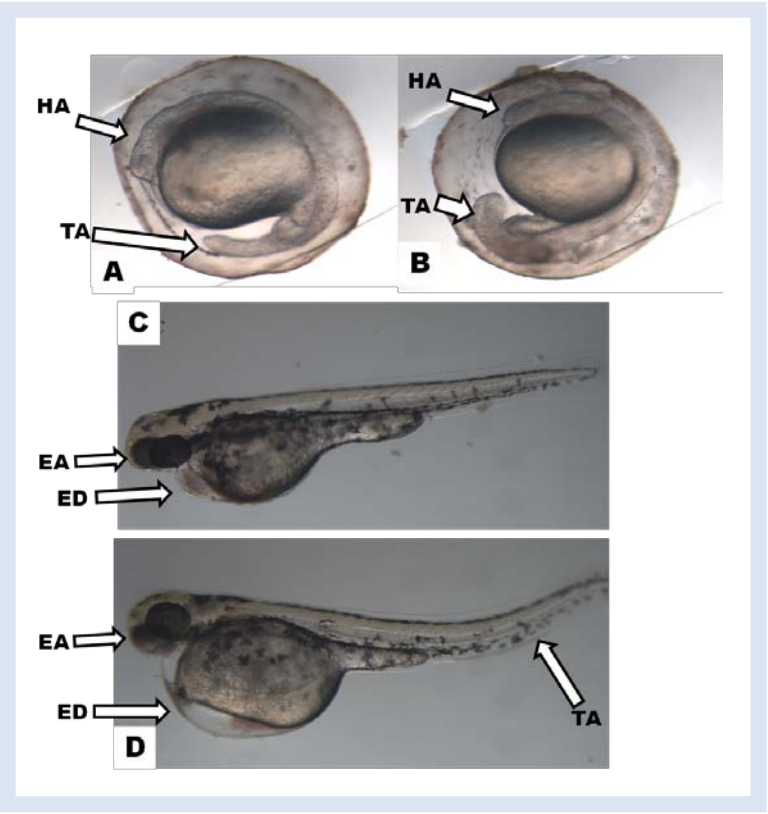

Fig.3.4.6. Zebrafish embryos microinjected with pure GAA. A and B, micrographs at 24, ,and C and D at 48 h pmi. Arrows indicate relevant morphological changes respect to normal development. HA, EA, TA and ED are head, eyes and trunk aberrations, and edema, respectively.

Since GAA is a precursor of CYN, microinjection of this toxin and crude extracts of the CYN^+ Nd GAA^+*A. ovaliporum* UAM-MAO strain were also tested. CYN solutions in sterile Milli-Q water were used at a final concentration of 2 and 20 μM. The crude extract was obtained by homogenizing lyophilized cell biomass in sterile Milli-Q water (10 μg of lyophilized biomass per mL water), and further centrifugation (6000 x*g* 10 min). The original extract and a 10-fold dilution of it were utilized. The approximate CYN concentration in the original extract was 10 nM.

Microinjection of CYN caused similar effects to GAA: a dose-dependent embryo death (Fig.3.4.7), and various morphological aberrations (Fig.3.4.8).

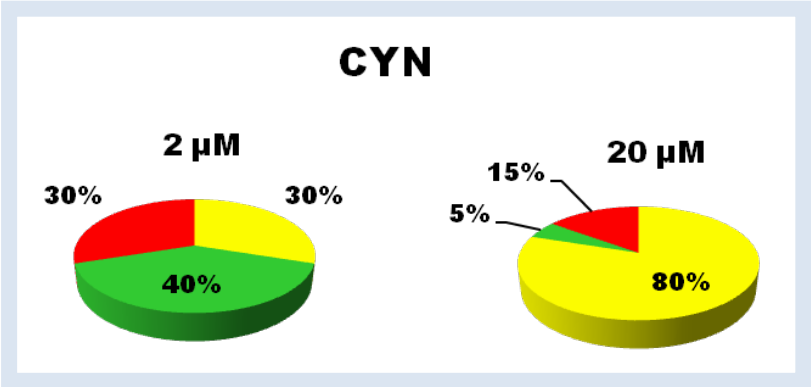

Fig.3.4.7. Effect of different CYN concentration on zebrafish embryos at 24 h pmi. Unaffected (green), abnormal (red) and dead (yellow).

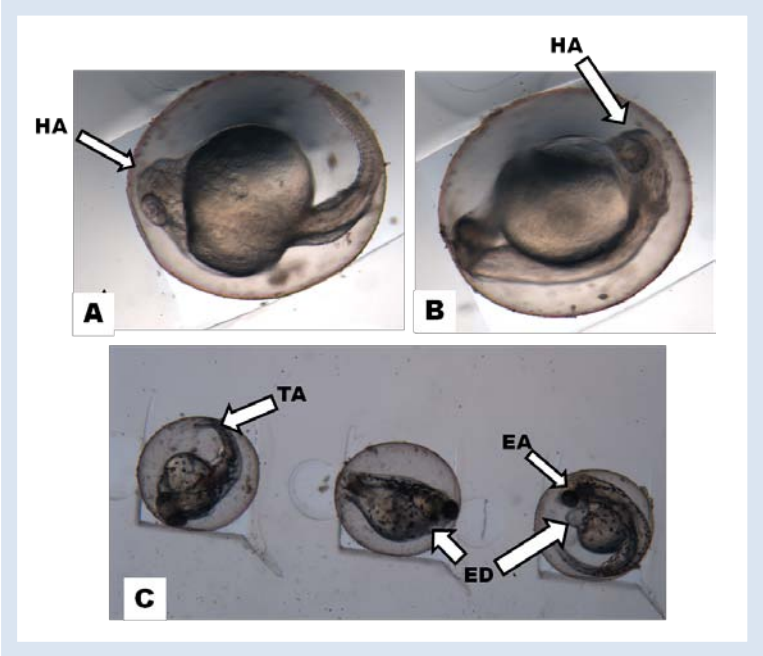

Fig.3.4.8. Zebrafish embryos microinjected with pure CYN. A and B micrographs at 24 h, and C at 48 h pmi, respectively. Arrows indicate relevant morphological changes respect to normal development. HA, EA, TA and ED are head, eyes and trunk aberrations, and edema, respectively.

The few experiments performed with crude extract showed that a large amount of embryos (near 80%) microinjected with the original extract died after 24 h. With the 10-fold diluted extract lower mortality (Fig.3.4.9 A) was observed, but significant development aberrations appeared already 24 h pmi (Fig.3.4.10). Therefore, it seems that *A. ovalisporum* extracts could affect zebrafish embryos in a dose-dependent manner

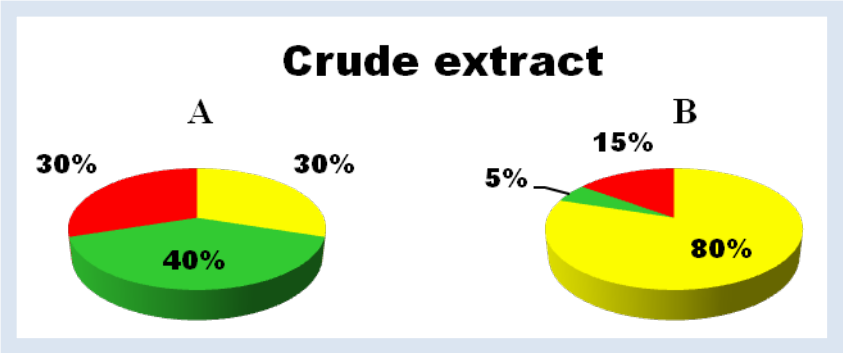

Fig.3.4.9. Effect of *A. ovalisporum* cell extract on zebrafish embryos at 24 h pmi. Unaffected (green), abnormal (red) and dead (yellow) embryos. A) 10-fold diluted extract; B) original extract (equivalent to a concentration within the embryo of ca. 0.2 nM CYN and 0.6 nM GAA).

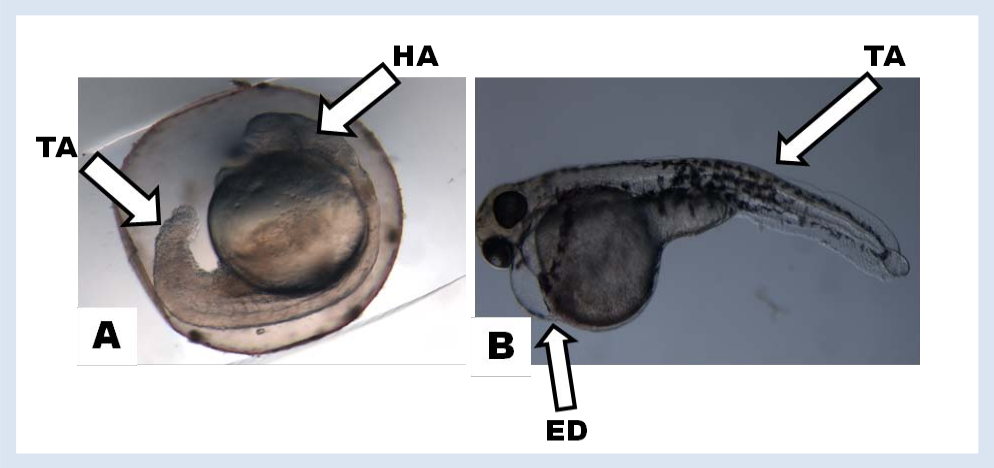

Fig.3.4.10. Zebrafish embryos microinjected with a 10-fold diluted extract. A and B, micrographs taken at 24 and 48 h pmi, respectively. Arrows indicate relevant morphological changes respect to normal development. HA, EA, TA and ED are head, eyes and trunk aberrations, and edema, respectively.

The toxicity by GAA in zebrafish embryos is pioneer; but the toxicity of microinjected CYN and *A. ovalisporum* crude extract was already reported by Berry et al. (2009). Our data confirmed this author idea, that other toxic compounds aside CYN are present in *A. ovalisporum*. As in our case, it was seen that CYN was only toxic when microinjected into the embryos, immersion not causing significant mortality or development impairment.

Acknowledgments

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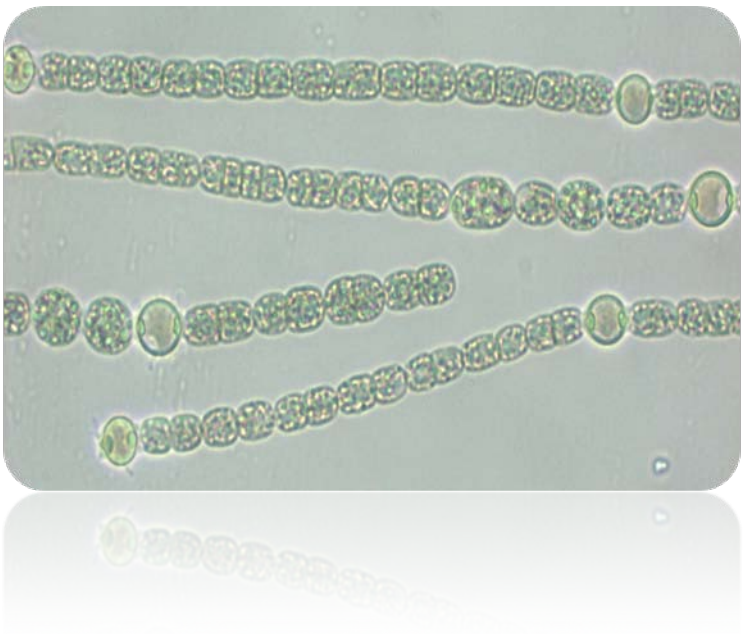
3.5. CHAPTER V

Influence of glycine and arginine on cylindrospermopsin production in *Aphanizomenon ovalisporum*



Influence of glycine and arginine on cylindrospermopsin production in *Aphanizomenon ovalisporum*

Manuscript in preparation

3.5.1. Abstract

Arginine (Arg) and glycine (Gly) seem to be the only substrates accepted by the amidinotransferase that catalyzes the first step of the synthesis pathway of the cyanotoxin cylindrospermopsin (CYN), leading to guanidinoacetate (GAA). Here, the effect of these amino acids on the production of CYN in cultures of the cylindrospermopsin-producing strain *Aphanizomenon ovalisporum* UAM-MAO has been studied. Arg clearly increased CYN content, the increment appearing triphasic along the culture. On the contrary, Gly caused a decrease of CYN, observable from the first day on. Interestingly, the transcript of the gene *ntcA*, key in nitrogen metabolism control, was also enhanced in the presence of Arg and/or Gly, the trend of the transcript oscillations being similar to that of *aoa/cyr*. The inhibitory effect of Gly in CYN production seems not to result from diminishing the activity of genes considered involved in CYN synthesis, since Gly, as Arg, enhance the transcription of genes *aoaA-C* and *cyrJ*. On the other hand, culture growth is affected by Arg and Gly in a similar way as CYN production, Arg stimulating and Gly impairing it. Taking together, our data show that the influence of both Arg and Gly on CYN changes seem not to be due to a specific effect on the first step of CYN synthesis; it rather appears to be the result of changes in the physiological cell status.

Keywords: *Aphanizomenon ovalisporum*, *aoa/cyr/ntcA* genes expression, guanidinoacetate, cylindrospermopsin, arginine, glycine.

3.5.2. Introduction

There is a correlation between eutrophication, climate change and toxic harmful algal blooms (HABs), in which cyanobacteria are significative components. High concentration of nitrogen (N) and phosphorous (P) contribute to the massive proliferation of toxic cyanobacteria in water reservoirs for consumption or recreational purposes (Paerl and Otten, 2013). Among the cyanotoxins, the potent alkaloid and protein synthesis inhibitor cylindrospermopsin (CYN) is of increasing concern, due to the growing number of world-wide detections reported in the last years (Kinnear, 2010).

Inorganic nutrient availability seems to play a key role in CYN production, since nitrogen, phosphate and sulphate starvations cause significant changes in the toxin production (Saker and Neilan, 2001, Bacsi et al., 2006, Shalev-Malul et al., 2008, Bar-Yosef et al., 2010).

Despite the large molecular information related to CYN production, data about the regulation of its synthesis is still scarce. Till date, all gene clusters associated to CYN synthesis (*aoa/cyr*) are highly similar along the entire gene regions. The CYN synthesis pathway model includes an amidinotransferase (AMDT), codified by the *aoaA/ cyrA* gene, that would catalyze the first step to produce guanidinoacetic acid (GAA). In the next steps, the following enzymes would be involved: a mixed enzyme complex constituted by a non ribosomal peptide synthetase (NRPS)-polyketide synthase (PKS), codified by *aoaB*/*cyrB*, a PKS codified by *aoaC /cyrC*, other PKSs, and tailoring proteins. AMDTs of the CYN-producing strains *Cylindrospermopsis raciborskii* AWT205 (CyrA) (Muenchhoff et al., 2010) and *Aphanizomenon ovalisporum* UAM-MAO (AoaA) (Barón-Sola et al., 2013) were cloned, overexpressed and biochemically characterized. Unlike the AMDTs described before, CyrA and AoaA show narrow substrate specificity, seeming to work well just with arginine (Arg) and glycine (Gly) as an amidino group donor and acceptor respectively. Besides, a complex mixed ping-pong kinetic mechanism is involved in the two cyanobacteria AMDTs.

A. ovalisporum is a bloom-forming cyanobacterium, all of its reported strains, except one, being CYN producers. Recently, it was described as a potential invasive species (Mehnert et al., 2010, Sukenik et al., 2012). Interestingly, it has been reported that different organic nitrogen compounds can be utilized by *A. ovalisporum* ILC146 strain,

dissolved organic nitrogen appearing to be the major N source in *A. ovalisporum* bloom episodes in Lake Kinneret (Berman, 1997). The role of organic nitrogen compounds, amino acids among them, on cyanobacterial growth and on primary and secondary metabolism has been deeply studied in various cyanobacteria species (Kapp et al., 1975; Flores and Herrero, 2005; Zubkov and Tarran, 2005; Pernil et al., 2008).

Being Arg and Gly the specific substrates of the cyanobacteria AMDTs responsible for the first step of CYN synthesis, we thought interesting to explore if these amino acids influenced CYN production. For that, the production of CYN and GAA in cultures of *A. ovalisporum* UAM-MAO grown in the absence and in the presence of Arg and/or Gly was compared. The effect of the amino acids in the expression of four genes involved in CYN formation, *aoaA–C* and *cyrJ*, were compared as well. Furthermore, it was also evaluated the influence of Arg and Gly in the expression of the gene *ntcA* that codifies for a key regulator of nitrogen metabolism, considering the high nitrogen content of CYN, and previous suggestion (Chapter IV) of a possible role of that gene in CYN production.

3.5.3. Materials and methods

3.5.3.1. Culture conditions

A. ovalisporum UAM-MAO strain (Barón-Sola et al., 2012) was used. Three independent experiments were performed with batch BG_{11} (Rippka et al., 1979) (pH 8; 20 mM HEPES) cultures, at 28 °C, continuous white light of 60 µmol photons m^{-2} s^{-1}, and constant aeration.

Four different culture conditions were assayed: a control, with nitrate as nitrogen source (BG_{11}), and three cultures supplementated with 1 mM Arg, Gly or both. Samples were withdrawn every 24 h during 9 days, and all of them analyzed in triplicate. Cell observations were done with an Olympus BH-2 microscope at 400x magnification equipped with a Leica DC300F digital system.

3.5.3.2. Growth parameters

Growth was followed by measuring the O.D. at 750 nm. Chlorophyll *a* (Chl_a) was also determined colorimetrically, essentially after Marker et al. method (1980).

3.5.3.3. Gene expression analysis

The transcription analysis of different genes (*aoaA-C, cyrJ* and *ntcA*) was carried out as described in Materials and Methods section of Chapter III.

3.5.3.4. CYN and GAA determination

CYN was determined as in Barón et al. (2012), and GAA as described in the Mateiral and Methods section of Chapter IV.

3.5.3.5. Data analysis

All data analyses were performed using GraphPad Prism® 5, and statistical analyses ANOVA (Bonferroni test), with significance level of $p < 0.01$, were performed using SPSS software® (version 19.0.0, 2011).

3.5.4. Results

3.5.4.1. CYN accumulation

Three phases in CYN accumulation were appreciated along the experimental time (216 h) in the three types of cultures utilized (Fig. 3.5.1). Initially, an increase of the toxin content was detected, followed by an intermediate phase with a CYN reduction and a final stage, with a new toxin rise. The duration and intensity of these phases were quite different between cultures.

In the presence of Arg (Fig. 3.5.1B), CYN content did not differed remarkably from the control (Fig.3.5.1A). Gly addition caused a significant increase of CYN just at 24 h, followed by an almost steady level (Fig 3.5.1C), with values always lower than the control. Arg plus Gly supplementation caused a slight increase in toxin content (Fig 3.5.1D), followed by several fluctuations till 120 h, when CYN rose constantly. In general, intracellular CYN was the main fraction, representing 65-90%; an exception was the glycine supplemented culture, in which intracellular fraction was only 30-50%. Remarkably, in this Gly culture the percentage of extracellular CYN rose in the last points assayed (7^{th} to 9^{th} day).

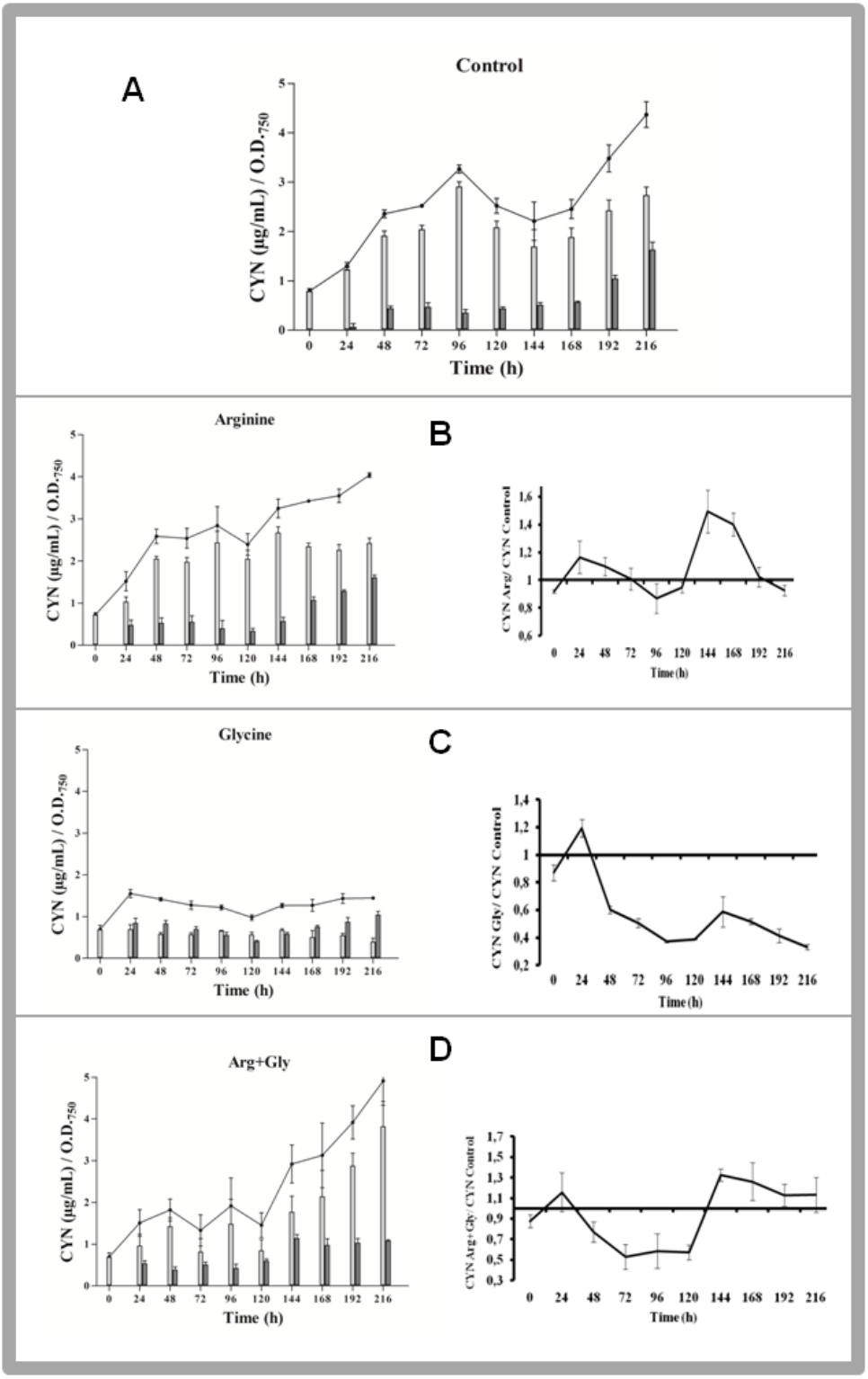


Fig.3.5.1. Effect of arginine and glycine on cylindrospermopsin content in *A. ovalisporum* UAM-MAO. A) Control culture; B) plus Arg; C) plus Gly; D) plus Arg and Gly. Total CYN is plotted in lines; intracellular and extracellularr CYN in light and dark grey bars, respectively. Values are the average of three replicates; error bars indicate ±SD from the mean (n = 3)..

3.5.4.2. Gene expression analysis

The expression of all genes analyzed was throughout the assay time significantly higher in the Arg culture than in the control (Fig. 3.5.2). The oscillation pattern of the *aoa* and *cyr* transcripts was similar. The transcription increased initially (from 0 to 48 h), then declined (till 72 h in *aoaC* and *cyrJ* or 96h in *aoaA-B*); later, it increased again, reaching the biggest value at 120 h; and finally, a gradual decrease was observed. This oscillatory pattern was different from that of CYN content (cf. Figs 3.5.1B and 3.5.2). In effect, CYN content in the Arg-supplemented medium increased ($p<0.01$) slightly respect to the control at 24, and largely about 150 h (Fig.3.5.1B). A small gene expression increase coincided with the first rise of CYN; but the second increase of gene expression took place when CYN was leveling off. The biggest CYN peak appeared 24h later than that of gene expression.

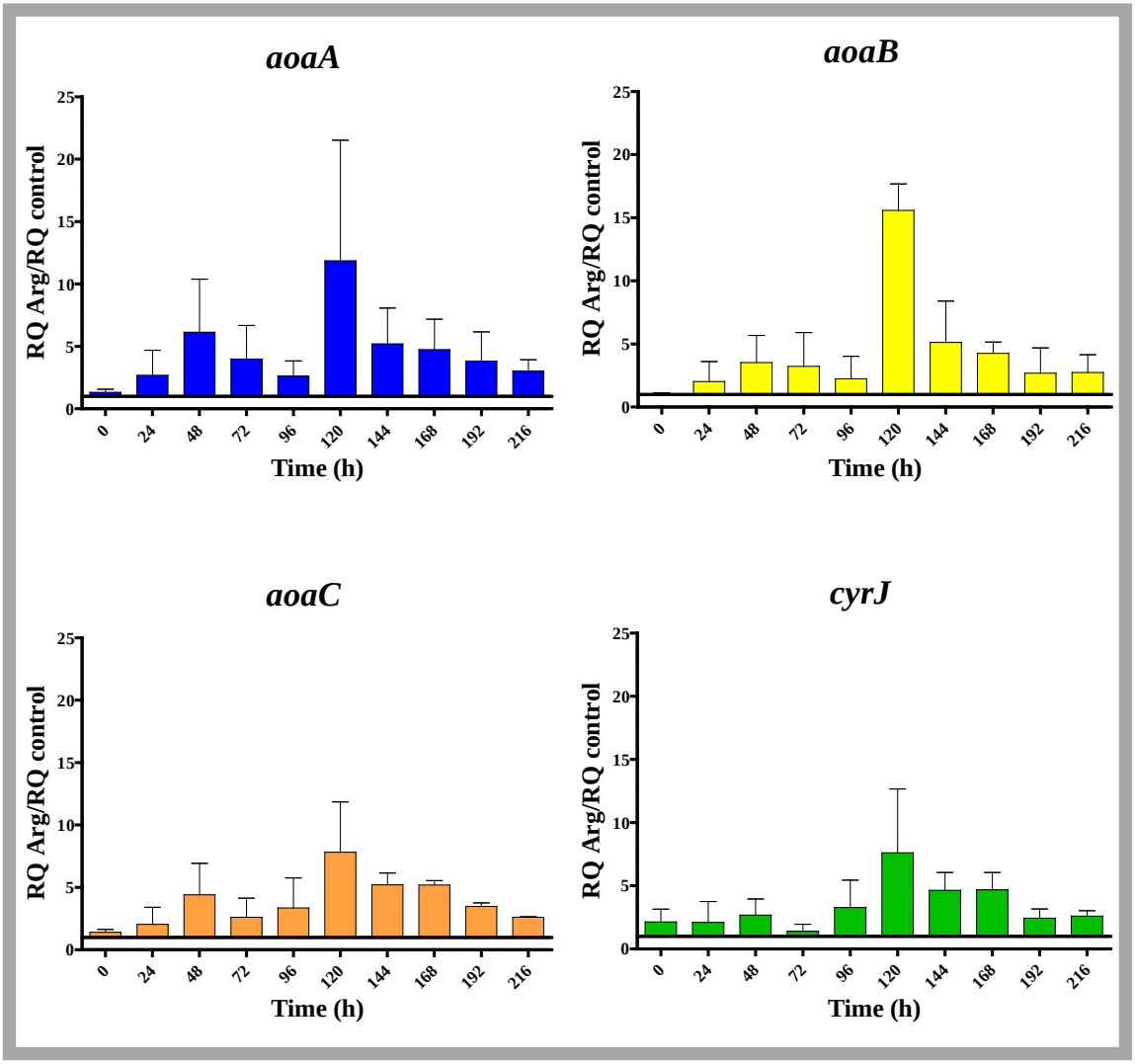


Fig.3.5.2. Relative expression of *aoaA, aoaB, aoaC* and *cyrJ* genes in *A. ovalisporum* UAM- MAO grown in BG_{11} medium supplemented with arginine. Data are presented as the ratio between expression levels in Arg supplemented (BG_{11} + Arg) and control culture (BG_{11}). The expression levels are relative to 16S rRNA gene. Error bars indicate standard deviations of three replicates.

In cultures supplemented with Gly alone, all gene transcripts increased with respect to the control (Fig. 3.5.3). Unlike Arg cultures, the increment pattern differed among the transcripts, especially that of *aoaB* gene. Thus, the maximum of *aoaA* , *aoaC* and *cyr* transcripts was at 48 h, while that of *aoaB* was at 192 h. The *aoaA* and *aoaC* expression also showed a high increment at 192 h. Curiously, *aoaB* expression maintained an almost circadian pattern. The effect of Gly on *aoa* genes transcription contrasts,

therefore, with that on CYN production (Fig. 3.5.1C), since the toxin content was significantly lower ($p<0.01$) than that of the control from 24 h to the end (216 h). Only at 24 h, the content was considerably higher in the Gly-supplemented culture than in the control.

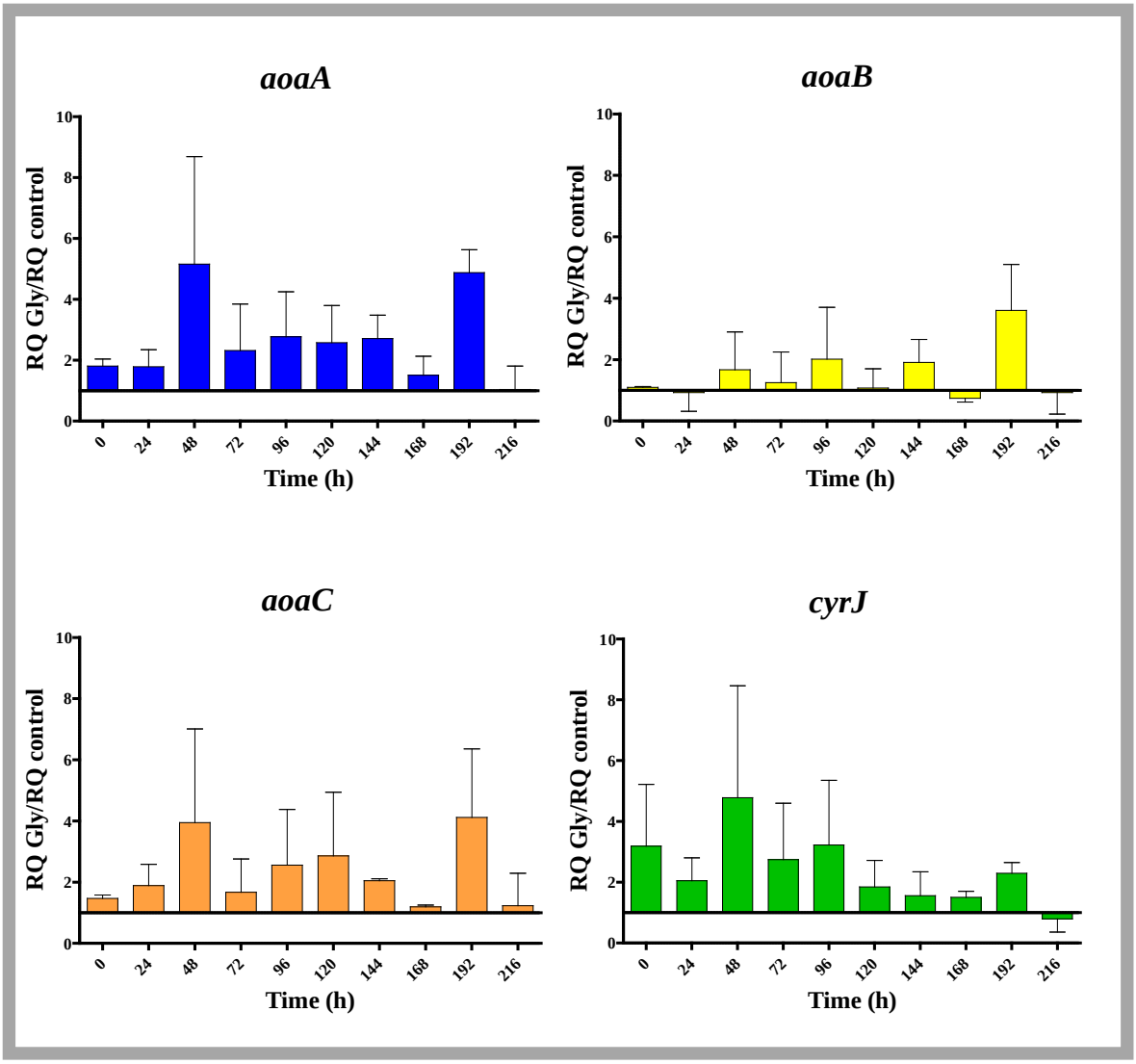


Fig.3.5.3. Relative expression of *aoaA*, *aoaB*, *aoaC* and *cyrJ* genes in *A. ovalisporum* UAM-MAO strain grown in BG_{11} medium supplemented with glycine. Data are presented as the ratio between expression levels in Gly supplemented (BG_{11} + Gly) and in the control culture (BG_{11}). The expression levels are relative to 16S rRNA gene. Error bars indicate standard deviations of three independent experiments.

In the presence of Arg plus Gly, *aoa* and *cyr* transcript levels were also bigger than in the control (Fig. 3.5.4). The fluctuations of all the transcripts were similar throughout

the time, with a maximum at 192 h. From 0 to 192 h a continuous up and down was observed; but there were differences between the genes, both with respect to time duration and intensity. Interestingly, the CYN content variations exhibited a distinct trend (Fig.3.5.1D). During the first half of the experiment a similar tendency to that of Gly treatment was observed, while during the second part, the pattern was closer to that of Arg-supplemented cultures.

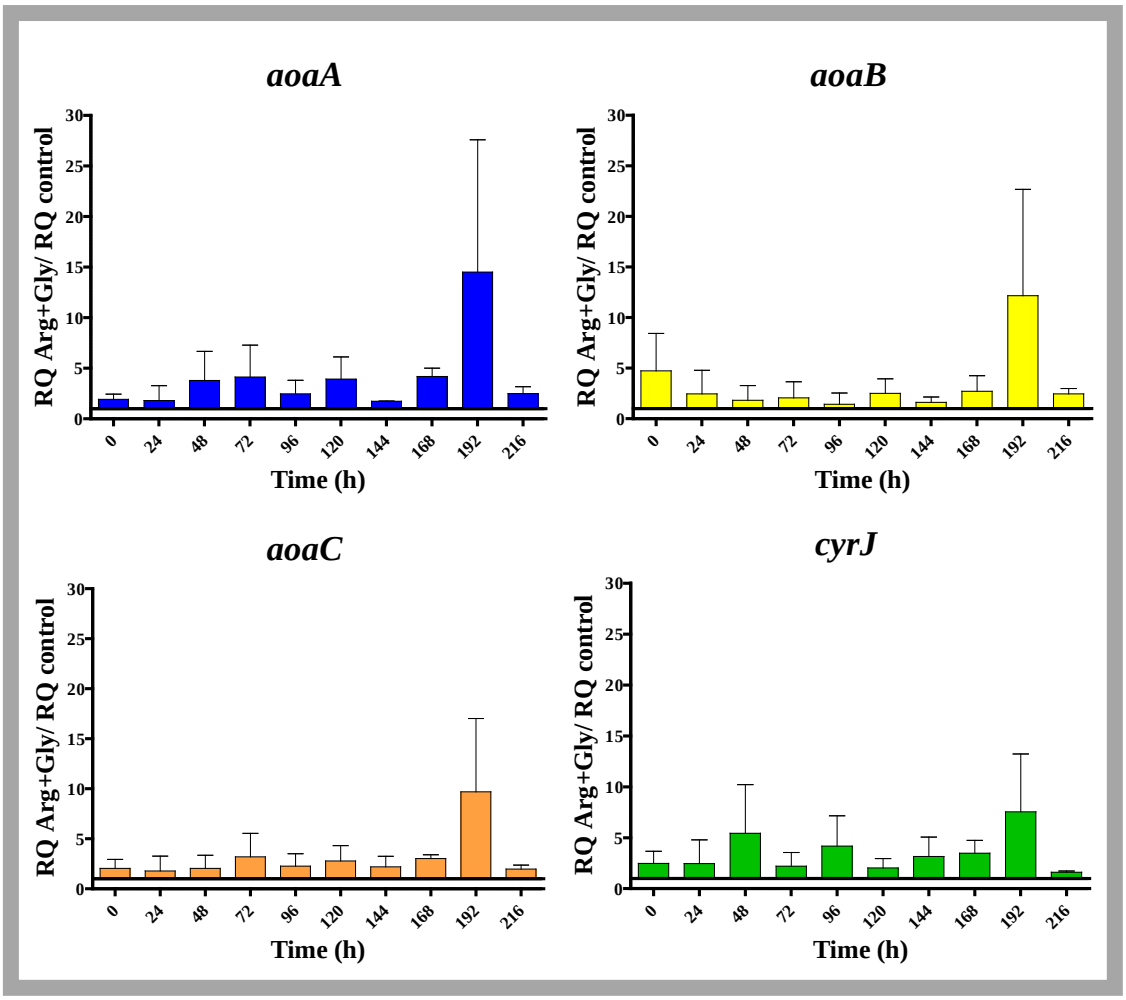


Fig.3.5.4. Relative gene expression of *aoaA*, *aoaB*, *aoaC* and *cyrJ* genes in *A. ovalisporum* UAM-MAO strain grown in BG_{11} medium supplemented with arginine and glycine. Data are presented as the ratio between the expression levels in the Arg plus Gly-supplemented culture and the control culture (BG_{11}). The expression levels are relative to 16S rRNA gene. Error bars indicate standard deviations of three replicates.

3.5.4.3. GAA accumulation

In all cultures, intracellular GAA varied along the time (Fig. 3.5.5). Except in the Gly culture, a gradual increase of GAA was observed from 0 to 96 h. Nevertheless, the rise slope as well as the maximum value attained differed. In the Gly culture, GAA rose abruptly within the first 24h. In all the cases, after the initial increase, a long decrease was observed until the penultimate day of the experiment (192 h). The GAA value remained in the Arg and Arg plus Gly cultures below the control till that day, when GAA content rose again conspicuously, above the control value. Interestingly, in all instances the concentration of GAA (amount per biomass unit) was 1.5 to 9 times higher than that of CYN. No correlation was observed between GAA fluctuations and transcription of any of the *aoa*/*cyr* genes (cf. Figs. 3.5.5 and 3.5.2- 3.5.4).

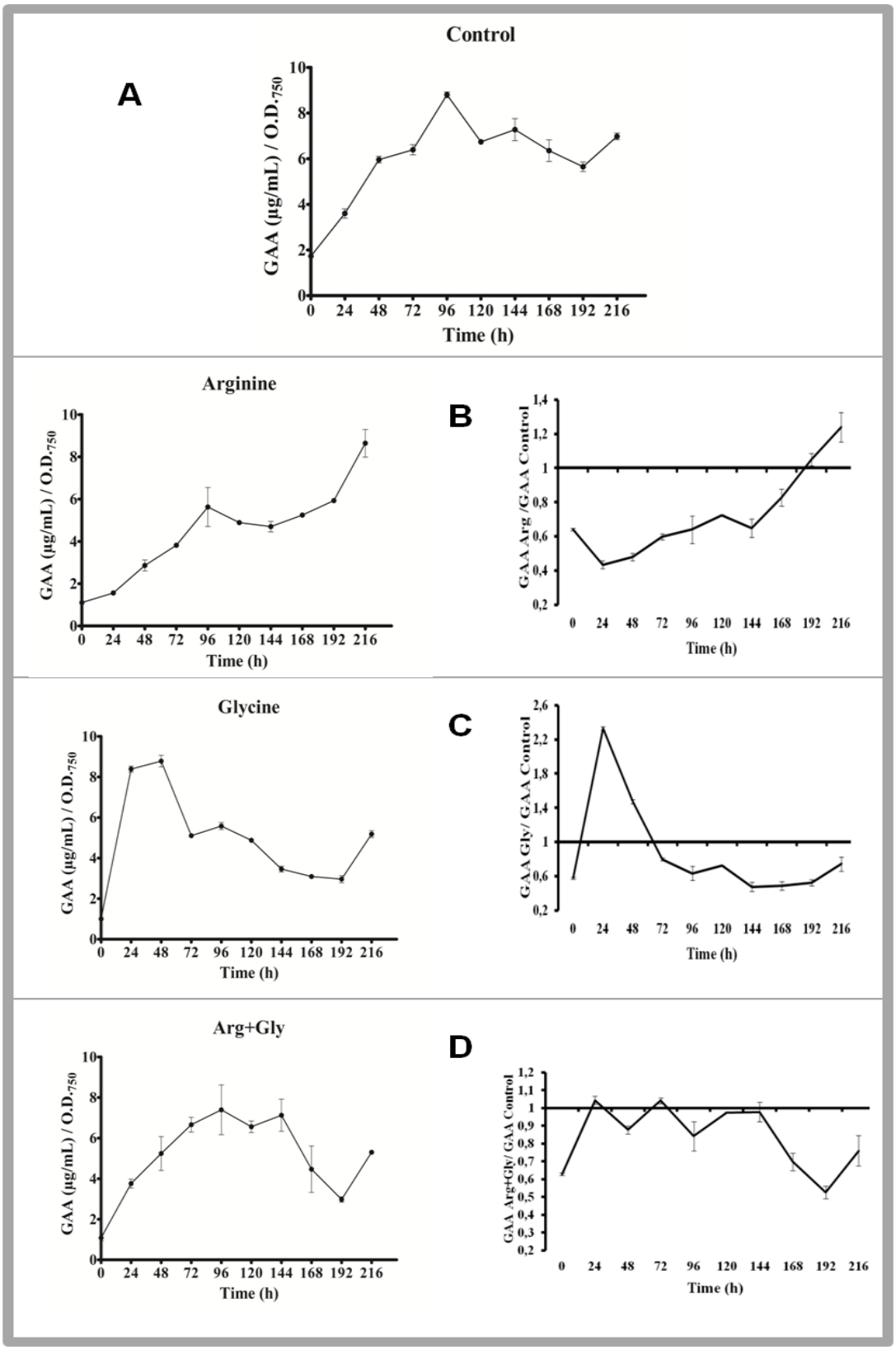


Fig.3.5.5. Effect of arginine and glycine on intracellular guanidinoacetate of *A. ovalisporum* UAM-MAO. A) Control culture; B) Arg-supplemented culture; C) Gly-supplemented culture; D) Arg plus Gly-supplemented culture. Values are the average of three replicates; error bars indicate ±SD from the mean (n = 3).

3.5.4.4. Gene expression analysis of ntcA

The expression of the nitrogen master control gene *ntcA* was enhanced throughout the time in all treatments assayed (Fig. 3.5.6). In every amino acid treatment gene expression changes were observed, but the changes were different in duration and magnitude, and depended on the particular gene. Interestingly, the trend of the *ntcA* transcript changes was similar to that of the *aoa/cyr* genes (cf. Figs. 3.5.6 and 3.5.2-3.5.4).

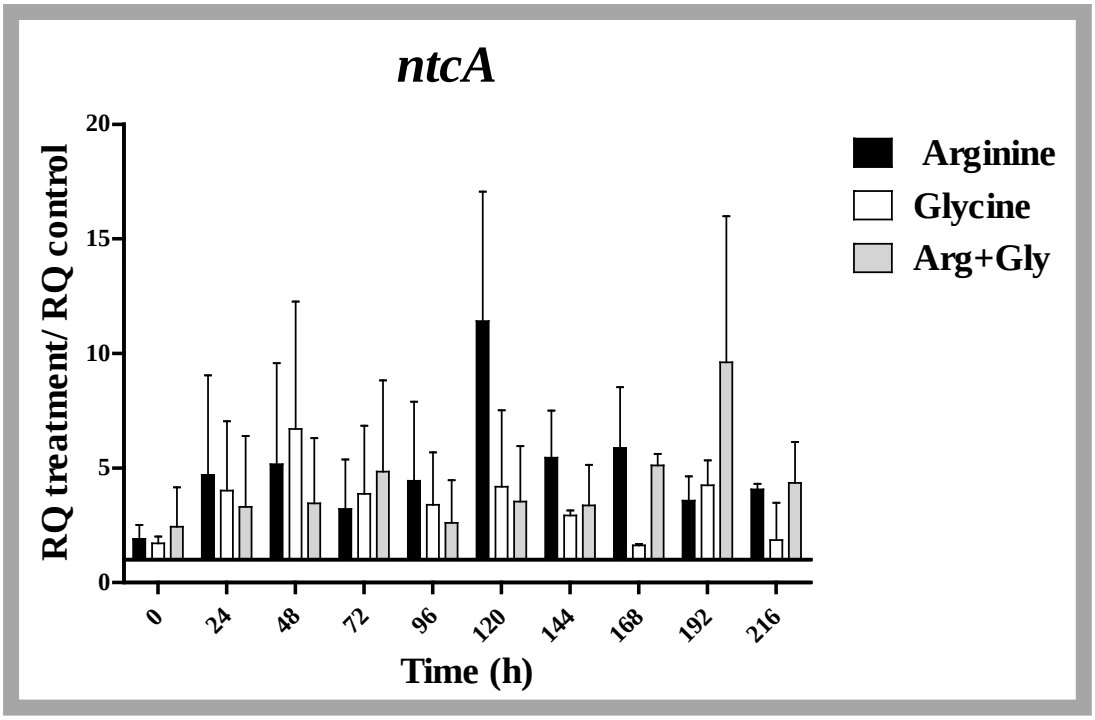


Fig.3.5.6. Relative expression of *ntcA* gene in *A. ovalisporum* UAM-MAO grown in BG_{11} medium supplemented with arginine and/or glycine. Data are presented as the ratio between the expression levels in the amino acid-supplemented cultures and the control culture (BG_{11}). The expression levels are relative to 16S rRNA gene. Error bars indicate standard deviations of three replicates.

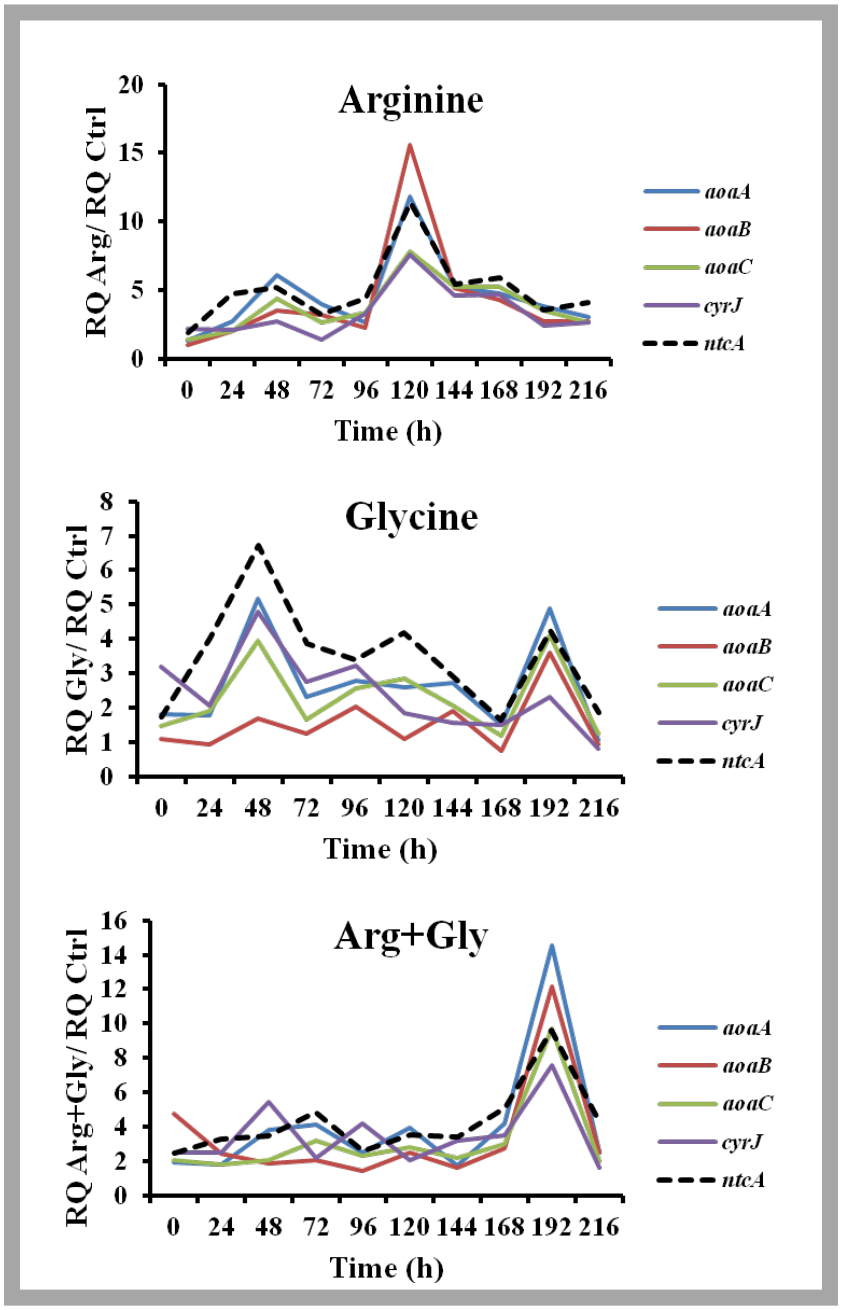


Fig.3.5.7. Relative expression of *aoaA*, *aoaB*, *aoaC*, *cyrJ* and *ntcA* genes in *A. ovalisporum* UAM-MAO strain grown in BG_{11} medium supplemented with arginine and/or glycine. Data are presented as the ratio between the expression levels in the amino acid-supplemented cultures and the control culture (BG_{11}). The expression levels are relative to 16S rRNA gene.

3.5.4.5. Effect of arginine and glycine on growth

Growth was followed by the absorbance at 750 nm and Chl_a concentration, but under the conditions used, the expected growth curve was only obtained considering the absorbance values. Therefore, the absorbance was the elected growth parameter.

The cultures with added Arg, either alone or with Gly grew significantly ($p<0.01$) better than the control or Gly cultures, the strongest stimulatory effect being when Arg was added alone (Fig. 3.5.8). Remarkably, the growth in the culture supplemented with Gly was the lowest in a significant way ($p<0.01$) during the first half of the assay (24 to 96h).

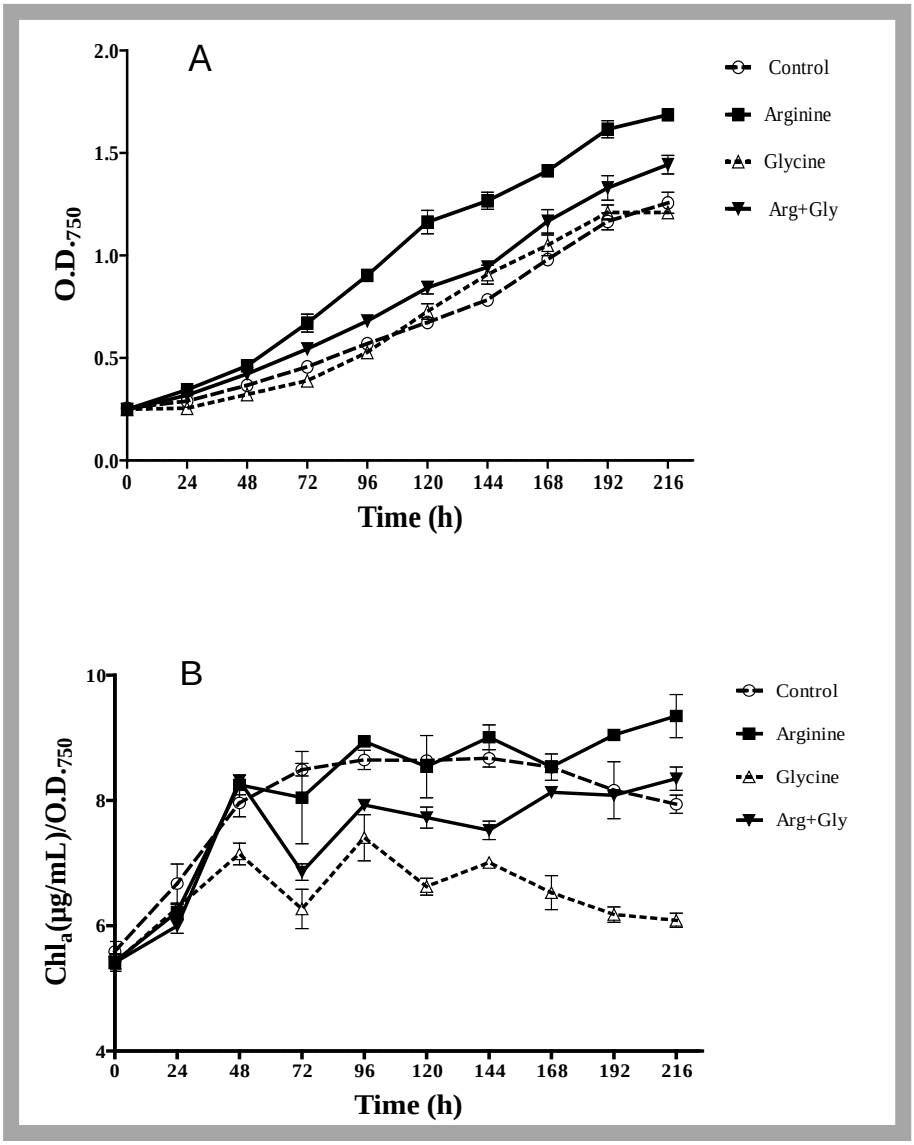


Fig.3.5.8. Effect of arginine and glycine on growth and chlorophyll concentration of *A. ovalisporum* UAM-MAO. Values are the average of three replicates; error bars indicate ±SD from the mean (n = 3).

Chl_a content was affected in a different way by the presence of Arg and Gly. The effect of Arg supplementation was not significant ($p<0.01$) till the two last samplings, when a stimulatory effect was observed. But, Gly clearly caused a considerable reduction in Chl_a concentration throughout the assay ($p<0.01$). The addition of both amino acids also decreased significantly ($p<0.01$) Chl_a concentration respect to the control, but the decrease was slighter than in Gly cultures.

3.5.5. Discussion

We have evaluated in *A. ovalisporum* UAM-MAO The influence of Arg and Gly in CYN production, considering that these amino acids could favor the synthesis of the toxin in two manners: first, by providing the unique substrates apparently recognized by the AMDT responsible for the GAA formation in the first step of CYN synthesis (Muenchhoff et al., 2010); and second, by enhancing available nitrogen, which would contribute to satisfy the high N needs for CYN synthesis. The experiments have been performed with batch cultures of the CYN^+ *A. ovalisporum* strain UAM-MAO, grown in a medium with NO_3^- as initial N source (BG11), supplemented or not with Arg, Gly or a mixture of both amino acids.

CYN content was affected in a different way by the presence of the amino acids: Arg clearly enhanced the production, while Gly decreased it (Fig 3.5.1) after the first 24 h. Both increase and decrease were concentration dependent (Data not shown). The inhibition by Gly was alleviated by the presence of Arg. The enhancement by Arg exhibited a curious triphasic pattern.

It appears that the influence by Arg and Gly in CYN production could not be attributed to effects on the activity of genes related to its synthesis, for the transcription of the *aoaA-C* and *cyrJ* genes are similarly increased by either of the two amino acids (Figs. 3.5.2-3.5.4).

Being *aoaA* the gene codifying for the AMDT that catalyzes the formation of GAA from Arg and Gly, special emphasis has put on comparing the effect of the two amino acids on both *aoaA* expression and GAA production. No correlation was found between the *aoaA* transcript levels and GAA cell content in the different cultures utilized (cf. Figs 3.5.5 and 3.5.2-3.5.4). Arg and Gly when added alone or together increased

significantly *aoaA* gene transcription throughout the experimental period; but GAA content was in the Arg significantly lower in all treatments respect to the control, except in part of the last growth period. GAA increased during the first 48 h upon Gly addition; later it leveled off, in parallel to CYN. Arg treatment caused different GAA increments along the culture, in a triphasic way alike to CYN (cf. Figs. 3.5.5 and 3.5.1). In spite of the basis found to think that the control by Arg and Gly of CYN and GAA production is not at a transcriptional level, other genes in *aoa* cluster from *A. ovalisporum* UAM-MAO should be analyzed.

It has been previously observed that different nitrogen sources affect CYN content (Saker and Neilan, 2001; Shalev-Malul et al., 2008; Stucken, 2010) and *aoa/cyr* expression levels (Shalev-Malul et al., 2008; Stucken, 2010), but no experiment to analyze the effect of amino acids was performed. It would be advisable to perform experiments with other CYN^+ strains from different species, as well as with other amino acids, to assess how the presence of a particular amino acid could condition CYN production.

Interestingly, the transcription of the gene *ntcA* was significantly enhanced respect to the control in the presence of all the amino acids assayed (Fig. 3.5.6), the trend of the transcription kinetics being similar that of *aoa/cyr* (Fig.3.5.7). That fact suggests, among other ideas, that *ntcA* and the genes responsible for CYN synthesis are controlled by a common regulator, and/or that *ntcA* controls *aoaA* and *cyrJ* expression. Previous analyses of *ntcA* transcription under different nitrogen sources showed that ammonium caused the repression of this gene, while other nitrogen compounds increased it (Lindell et al., 1998, Lindell et al., 2002). Besides, the assimilation of different organic nitrogen compounds is controlled by *ntcA* (Collier et al., 1999). Further experiments are needed to clarify how Arg and Gly modulate in a similar way the activity of *ntcA*, *aoaA-C* and *cyrJ* genes.

The influence of both Arg and Gly on CYN in *A. ovalisporum* UAM-MAO appears to be the result of changes in the physiological cell status, since its growth was remarkably favored in the presence of Arg, and reduced with Gly. Besides, with the mixture of both amino acids an intermediate growth effect was observed, as it occurred with CYN production (cf. Figs. 3.5.8 and 3.5.1).

The growth stimulation by Arg was not a surprise, since it was already established that amino acids and other organic nitrogen sources can be used by cyanobacteria as a source of N and C (Kapp et al., 1975; Vaishampayan, 1982; Herrero and Flores, 1990). With respect to utilization of Arg by *A. ovalisporum*, it was also reported that dissolved organic nitrogen was the main N source utilized by a strain of this species in a bloom episode in Lake Kinneret, and that other organic nitrogen compounds, including some amino acids, could serve for its growth (Berman, 1997). Amino acid transporters with high and low affinity for Arg have been described in detail in the filamentous heterocyst-forming cyanobacteria *Anabaena* sp. PCC 7120 (Herrero and Flores, 1990). Besides, we have observed a significant reduction of heterocyst density in *A. ovalisporum* UAM-MAO (data not shown) when Arg was used as the only N source. It has been also demonstrated that simultaneous assimilation of amino acids (including arginine), ammonium and nitrate can take place (Bhattacharya et al., 2002). Moreover, large intracellular accumulation of Arg was described in *Synechocystis* sp. 6803 (Labarre et al., 1987) suggesting an important physiological role of this amino acid in cyanobacteria apart from its role as source for nitrogen reserve forming cyanophycine with aspartate.

Growth stimulation and reduction of *A. ovalisporum* UAM-MAO by Arg and Gly, respectively, seem to be dose dependent (data not shown). When planned this work, toxicity by Gly was unexpected; however, amino acid toxicity has been reported in several cyanobacteria (Hall and Jensen, 1980;Vaishampayan, 1982; Labarre et al., 1987; Herrero and Flores, 1990). Specifically, Gly accumulation has been described as toxic for *Synechocystis* sp. strain PCC6803 (Eisenhut et al., 2007). However, the mechanism of Gly toxicity remains unknown. This amino acid acts as a potent chelating agent for bivalent cations (Stevens et al.1995) and Eisenhut et al. (2007) observed that addition of $MgCl_2$ to the medium reduced glycine toxicity, suggesting that Gly reduces the amount of bivalent cations, particularly Mg^{2+}. That could be the reason of the significant lowering of Chl_a content in the Gly and Arg+Gly treatments (Fig 3.5.8)

Taking together, the herein data reveal that CYN and GAA production are clearly affected by the presence in the medium of Arg and Gly, and that the effects produced could be the result of the amino acid metabolism, and not necessarily of a direct oaction on AMDT synthesis.

Acknowledgments

We would like to thank Professor Julian de la Horra Navarro from the Departamento de Matemáticas of Universidad Autónoma de Madrid for their help in statistical analysis, and Dr. Rosa Sedano and Eva Martín, from the SIDI-UAM, for helping with LC-ESI-MS analysis.

4. GENERAL DISCUSSION

GENERAL DISCUSSION

Toxic cyanobacteria blooms in water bodies of different type are increasing in the last years; hence, the convenience and even necessity of monitoring at least water bodies used for water consumption and recreational use. Toxic cyanobacteria cannot be discriminated by morphological criteria. Undoubtedly, the assessment of cyanotoxicity should be through bioassays, but the agencies in charge of controlling water control encounter numerous difficulties to utilize bioassay techniques. Besides, it is difficult that bioassays render the immediacy needed to set up the measures to eliminate the causing-toxicity agents. Thus, the development of new tools to help to prevent cyanotoxicity risk is really convenient. The detection of toxic cyanobacteria by molecular means is one of those tools, due to its simplicity, rapidity and even reasonable price.

Our laboratory has been working for several years on developing reliable methods to detect potentially toxin-producing cyanobacteria, based on the simultaneous amplification of sequences of various genes involved in the synthesis of the toxins. Initially, the research was focused in MC-producing cyanobacteria (Ouahid et al., 2005 and 2009). In the present work, we wanted to extend the detection to CYN-producing strains, having as a final objective the joint detection in environmental samples of MC^+ and CYN^+ cyanobacteria. We have succeeded, by developing a multiplex assay to amplify simultaneously sequences of *aoa*, *cyr*, and *mcy* genes, as described in Chapter I.

The multiplex PCR assay allows avoiding or at least minimizing the occurrence of false negative results, because more than one single genetic marker increases the reliability of the method. The method goes well by using directly cells and environmental samples as DNA template, allowing in this way to avoid the DNA purification step. We think that the new methodology could be useful to detect CYN and MC-producers in blooms with mixed cyanobacteria populations. The technique could help to prevent the risk caused by MCs and CYN, if used before bloom formation is consolidated, by alerting on the presence, if there were, of MC and CYN producers, well before toxin releasing from cells into the surrounding water (Chapter I).

Another objective of this Thesis was to advance in the knowledge of CYN synthesis and net production, due to the increasing importance of this toxin (Kinnear, 2010). Despite the imposibility to obtain so far a knocked out mutant in CYN^+ strains other approaches have permit to propose a model for CYN biosynthesis (Kellmann et al., 2006, Mihali et al., 2008). The first results on CYN synthesis, with feeding experiments, clearly indicated that the precursor of CYN was GAA, originated from glycine that received an amidino group from an unknown donor (Burgoyne et al., 2000). This feature was later reinforced by genetical studies that described a series of genes (*aoa*/*cyr*), most likely involved in CYN synthesis. Based on these genetical data a complete model for CYN synthesis was first proposed by Mihali et al. (2008). Especially relevant was first the identification of a gene, named *aoaA* in *A. ovalisporum* (Shalev-Alon et al., 2002) and *cyr* in *C. raciborskii* (Mihali et al., 2008) and other species, putatively encoding an amidinotransferase that would be responsible for the initial step of CYN synthesis. As described in Chapter II, we have found that, indeed *aoaA* codifies in *A. ovalisporum* for an AMDT that catalyzes the amidination of glycine by arginine. The protein has been characterized, showing high amino acid homology to the first cyanobacterial AMDT studied, CyrA , from *C. raciborskii*, (Muenchhoff et al., 2010), and that also gives rise to GAA. Narrow substrate specificity is one of the features to distinguish both. AoaA, and CyrA from other AMDTs of non cyanobacterial origin (Fritsche et al., 1997; Humm et al., 1997b; Lee et al., 2002). Another distinct feature of AoaA and CyrA from other AMDTs is the mixed sequential/ping-pong enzyme mechanism of their activity (Fritsche et al., 1997 and 1998; Humm et al., 1997a; Lee et al., 2002). Additional characteristic of cyanobacterial AMDTs is the inhibition by the reaction products L-ornithine and GAA, suggesting a negative feedback control of the first step of CYN synthesis.

We have observed that GAA is accumulated in different type of cyanobacteria, especially in the strain of *A. ovalisporum* utilized in this work, UAM-MAO (Chapter III). Our interest in accumulation of GAA was initially mainly due to its well documented toxic character (Mori et al., 1996; Neu et al., 2002; Zugno et al., 2008).We cannot decide at this moment if all the GAA content in *A. ovalisporum* UAM-MAO is the result of the AoaA activity. On the other hand, the fact that GAA accumulates in strains that do not produce CYN leads to think of several hypotheses. One could be that

GAA is a cyanobacterial metabolite involved in processes different from CYN synthesis; therefore, its formation should proceed through a pathway, where an AMDT different from AoaA would act. An alternative hypothesis is that GAA could be formed by AoaA, but synthesis of CYN would not proceed further, due to the lack of the required genes for CYN synthesis or factors regulating the activity of those genes.

Till now, most cyanotoxicity studies have shown that cyanobacterial crude extracts are more toxic than the purified known toxins obtained from those extracts, MCs (Puerto et al., 2010) or CYN (Berry et al., 2009; Sieroslawska, 2013), suggesting that other unknown compounds are contributing to the noxious extract activity. Taking into account the abundant data on toxicity of GAA and our results on GAA accumulation, we think that GAA could contribute to cyanobacterial toxicity. We have started to explore this possibility by assaying GAA toxicity on embryos of zebrafish (*Danio rerio*), a model organism in vertebrate development (Chapter IV, Complementary work). As in CYN toxicity bioassays using zebrafish embryos (Berry et al., 2009), immersion experiments did not show toxic effects; but, microinjection assays revealed that GAA could produce deleterious effect along the different development stages, the toxicity being dose-dependent.

As a contribution to get insight into the control of CYN production, one chapter of this Thesis is devoted to explore the relationship between CYN production and the expression activity of genes related to its synthesis, *aoaA-C* and *cyrJ*, and nitrogen metabolism, *ntcA* (Chapter IV). The understanding of CYN synthesis regulation, both at a transcriptional and posttranscriptional level, is an interesting issue from an academic point of view, but also from a practical one. Indeed, it is critical to determine environmental factors that favor bloom formation and/or cyanotoxin production, which should be known for a correct risk assessment of contaminated waters.

There have already been reports on the influence of diverse environmental factors on CYN production (Saker and Neilan, 2001; Bacsi et al., 2006; Shalev-Malul et al., 2008; Preussel et al., 2009; Stucken, 2010; Vasas et al., 2010; Cires et al., 2011), but the lack of standardization and the discrepancy between results impede drawing a general conclusion.

We have analyzed in *A. ovalisporum* the relationship between the CYN content (extracellular and intracellular) and the expression levels of the genes involved in toxin synthesis. Intracellular fraction was in all cases the main toxin fraction, in agreement to the majority of previously reported data with *A. ovalisporum*. Under optimal growth conditions, a good correlation between total CYN content and transcript levels of the *aoa/cyr* genes over the time were observed in. Interestingly, the pattern of *ntcA* transcript oscillations was similar to those of *aoaa/cyr* genes. Besides, NtcA binding sequences along the *aoaA-C* cluster were found, as previously reported in the *cyr* gene cluster of *C raciborskii* CS-505 (Stucken, 2010), suggesting that nitrogen metabolism may control CYN synthesis. That would be a logic regulation due the high N demand for the synthesis of the alkaloid skeleton of CYN.

The presence of arginine and glycine, both being a N source and substrates of the cyanobacterial AMDTs, CyrA and AoaA, affect CYN and GAA production by *A. ovalisporum* UAM-MAO (Chapter V); however, each amino acid does it in a different manner. Thus, Arg promotes the production of the two compounds, but Gly diminishes it. Curiously, the two amino acids stimulate the transcription of *aoaA-C*, *cyr J*, and *ntcA* genes. Growth and chlorophyll *a* content is affected by Arg and Gly in a similar way to CYN content. Taking together, our data suggest that CYN and GAA production can be regulated postranscriptionally, and that the cell physiological status plays a key role in the production of the two compounds. The amino acid effects observed in our experiments could occur in nature, since dissolved organic nitrogen has been described as an important source of carbon and nitrogen for cyanobacterial growth in aquatic ecosystems (Berman, 1997; Berman and Bronk, 2003; Glibert et al., 2004). Furthermore, all cyanobacterial strains studied to date present transporters for organic nitrogen molecules including amino acids.

5. CONCLUSIONS/CONCLUSIONES

CONCLUSIONS

1. A multiplex PCR assay was developed to detect in a joint and reliable manner potentially microcystin and/or cylindrospermopsin-producing cyanobacteria, by using *mcy* and *aoa* sequences as molecular determinants. The method is effective even with phytoplankton biomass as DNA template. Therefore, it could contribute to foresee cyanotoxicity risk.

2. The amidino transferase of *A. ovalisporum*, AoaA, thought to be responsible for the first reaction of cylindrospermopsin synthesis, was overexpressed in *E. coli*, purified and biochemically characterized. It catalyzes guanidinoacetate (GAA) and ornithine formation from arginine and glycine in a sequential/ping-pong mechanism, GAA appearing as a non-competitive inhibitor of the reaction. The activity is significantly altered by some inorganic cations. The amino acid sequence of AoaA is highly similar to the previously described AMDT from *Cylindrospermopsis raciborskii* (CyrA), but the two proteins differ in several aspects, AoaA being active and stable in a wider range of pH and temperature activity and stability than Cyr.

3. GAA can accumulate in cells of different cyanobacteria types. GAA is also found in the surrounding medium. Being GAA a well-known toxic compound, its accumulation could contribute to cyanotoxicity.

4. CYN production does not seem to be solely or mainly controlled at a transcriptional level, since there is no correlation between the toxin production and *aoa/cyr* transcript levels.

5. Arginine and/or glycine affect CYN production and growth of *A. ovalisporum* in a distinct way. Arginine enhances CYN content and growth, while glycine decreases them. Both amino acids increase the transcription of *aoa/cyr* genes, and of the nitrogen master controller *ntcA* in a similar fluctuating manner, suggesting a relationship between the said genes.

CONCLUSIONES

1. Se ha desarrollado un método para detector de forma conjunta y fiable cianobacterias potencialmente productoras de microcistinas y/o cilindros-permopsina (CLP), utilizando secuencias *mcy* y *aoa* como determinantes moleculares. El método es efectivo incluso utilizando como fuente de ADN biomasa fitoplanctónica.

2. La amidinotransferasa de *Aphanizomenon ovalisporum*, AoaA, se ha sobreexpresado, purificado y caracterizado bioquímicamente. Cataliza la formación de guanidinoacetato (GA) y ornitina a partir de arginina y glicina según un mecanismo secuencial/ ping-pong. La actividad se inhibe de forma no competitiva por GA y se afecta por varios cationes inorgánicos divalentes. La secuencia aminoacídica de AoaA es muy pareciada a la AMDT ya descrita de *Cylindrospermopsis raciborskii* (CyrA); pero las dos proteínas difieren en algunos aspectos ya que AoaA es activa y estable en un intervalo de pH y temperatura mayor que CyrA.

3. El GA se acumula en cianobacterias de diferente tipo. También se encuentra en el medio externo. Al ser GA un reconocido compuesto tóxico, su acumulación podría contribuir a la cianotoxicidad.

4. La producción de CLP no parece estar controlada sólo o principalmente a nivel transcripcional ya que su producción y los niveles de expresión de los genes *aoa/cyr* no guardan correlación.

5. La presencia de arginina y glicina afecta de manera diferente la producción de CLP y el crecimiento de *A. ovalisporum* UAM-MAO. La arginina aumenta el contenido de CLP y el crecimiento, mientras que la glicina los reduce. Los dos aminoácidos aumentan la transcripción de genes *aoa*/*cyr* y del regulador maestro *ntcA* de una manera oscilante similar, lo que sugiere una relación entre dichos genes.

6. REFERENCES

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